Cold-induced photoinhibition, pigment chemistry, growth and nutrition of *Eucalyptus nitens* and *E. globulus* seedlings during establishment

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Schools of Agricultural and Plant Science and

CRC for Sustainable Production Forestry

University of Tasmania

Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

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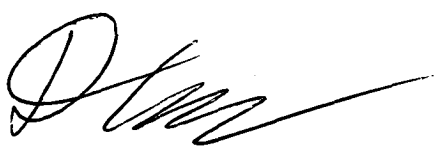

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Abstract

Australia is aiming to treble plantation wood production by 2020. *Eucalyptus globulus* Labill. and *E. nitens* (Deane and Maidem) Maiden are the predominant plantation species in southern Australia. This thesis describes physiological strategies employed by these species in response to cold-induced photoinhibition during seedling establishment. A series of experiments was conducted on seedlings pre-hardened in the nursery. Their physiological and growth responses after planting in the field was investigated.

A field trial was established at 350 m asl in early spring 1997. Severe cold-induced photoinhibition caused photodamage which restricted growth of non-hardened *E. globulus*. Artificial shading alleviated cold-induced photoinhibition and photodamage in both *E. globulus* and *E. nitens*, and increased growth in *E. globulus*. Before planting, nutrient-starved *E. nitens* were photoinhibited and had high anthocyanin levels. Increased photoinhibition was not measured after planting because of sustained xanthophyll activity and/or light attenuation by high anthocyanin levels. In other treatments changes in anthocyanin levels were related to the severity of cold-induced photoinhibition.

Relative to *E. nitens*, growth of *E. globulus* was more affected by cold-induced photoinhibition and photodamage. This was possibly due to inherently low levels of carotenoids and lack of acclimation to cold temperatures. The effects of shading on *E. globulus* and the absence of any effect of cold-hardening on *E. nitens* stresses the importance of incident light and pigment levels in cold-induced photoinhibition.

In a second field trial, an early winter planting of *E. nitens* was established at 700 m asl in June 1998. Shading may have increased biomass production because of alleviation of cold-induced photoinhibition. Growth in non-shaded than shaded seedlings was greater overall due to higher biomass production in spring and summer. Seedlings grew taller when shaded due to apical dominance. Fertilised seedlings produced more biomass in the field than non-fertilised seedlings.

Low growth rates of *E. nitens* during winter at 700 m asl were associated with high NPQ and sustained xanthophyll activity; photooxidation of chlorophylls, xanthophylls and ß-carotene (which decreased light absorption), and increases in lutein and neoxanthin (which indicated an antioxidant role). In general fertilised seedlings had higher pigment levels which maintained higher levels of light utilisation and dissipation. A controlled environment experiment which induced cold-induced photoinhibition, confirmed that galloylglucoses and flavonoids can act as antioxidants during seedling establishment. Sideroxylonals were also implicated in this role. Anthocyanin kinetics during seedling establishment indicated absorption of irradiance between 400 and 590 nm during periods of greatest cold-induced photoinhibition.

Chemical fractionation of leaf N and P indicated that *E. globulus* is more efficient at acquiring N and P than *E. nitens*. After planting, re-translocation of stored foliar protein N and inorganic P to roots occurred in both species. Greater amounts of re-translocation in fertilised seedlings may contribute to their superior growth in the field.

Planting seedlings involves risk. Planting nutrient-starved seedlings may decrease the risk of severe cold-induced photoinhibiiton and photodamage. However, this is at the expense of optimal growth performance. Planting altitude and season will determine whether fertilised or nutrient-starved seedlings should be planted.

Acknowledgements

During the course of this project I was supported by an Australian Postgraduate Award (APA) and a Co-operative Research Centre for Sustainable Production Forestry (CRC-SPF) scholarship. Substantial in-kind support was provided by North Forest Products (NFP) and North Eucalypt Technologies (NET) in the form of experimental sites, plant material and technical assistance.

Many people have helped make this project a success:

- Dr. Chris Beadle from whom I have learnt so much – thankyou;
- Dr. Noel Davies for generous and expert assistance with HPLC and MS analysis;
- Drs. Phil Brown, Mark Hovenden and Greg Holz for advice and support whenever requested;
- Dr. Steve Wilson for advice during the first year of my project;
- Ms. Ann Wilkinson and Mr. Petr Otahal for laboratory analysis assistance far beyond the call of duty;
- Mr. Dale Worledge for weather station construction and operation, and electric fence engineering;
- Dr. David Ratkowsky for expert statistical advice;
- Ms. Maria Cherry and Mr. Sven Ladiges for assistance in field measurements;
- Mr. Kelsey Joyce for assistance with frost tolerance testing, Mr. Chris Barnes for weed control advice, Ms. Renate Van Riet and Mr. Tim Hingston for seedling managment at the Ridgley nursery, Mr. Peter O'Neill and Mr. Terry Williams for seedling planting and tree shelter erection and Mr. Terry Williams for his excellent

assistance in the construction of the electrified fence, all of North Eucalypt Technologies;

• Messrs. Ian Ravenwood, Gavin Raison and Andrew Moore of North Forest Products for technical support at the North Forest Product's Somerset nursery;

• Mr. Ian Cummings and Ms. Tracy Jackson for technical support at the plant science glasshouse complex;

• Messrs. Phil Andrews and David Wilson for technical support at the agricultural science horticultural research station;

• Ms. Karen Barry for the use of tetra-galloylglucose standard data;

• Drs Bart Eschler and William Foley for sideroxylonal standard;

• Mr. Trevor Bailey of the Australian Antarctic Division for the loan of their Pro 200 homogeniser;

• Mr. Philip Coles for computers systems support;

• Ms. Tara Simmul for manufacturing the porous cloth bags;

• Mr. and Mrs. Greame Jones for providing accommodation in Burnie;

• Mr. Allan Mills and Professor Robert Menary for expertly fuelling my interest in plant physiology.

Primarily I have to thank my family: Owie (Kietha) Scarr, Rob, Gail, Joc (Oliver), Rundy (David) and Bazza (Harriet) Close for being my family, particularly Rob whom assisted with time management and as a general sounding board and Owie for the many stimulating lunch time conversations.

Kate Sice, who put up with the most, thankyou for being there.

Publications

Refereed journal articles

Chapter 3:

Close, D.C., Beadle, C.L., Brown, P.H. and Holz, G.K. (2000). Cold-induced photoinhibition affects establishment of *Eucalyptus nitens* (Deane and Maiden) Maiden and *E. globulus* Labill. Trees: Structure and Function 15: 32-41.

Chapter 7:

Close, D.C., Davies, N.W. and Beadle, C.L. Physiological antioxidants and compounds deterring herbivory and in light attenuation? Australian Journal of Plant Physiology 24: (In press).

Chapter 9:

Close, D.C., Beadle, C.L., Holz, G.K. and Ravenwood, I.C. (1999). A photobleaching event at the North Forest Products' Somerset nursery reduces growth of *Eucalyptus globulus* seedlings. Tasforests 11: 59-68.

Manuscripts for refereed journals in internal CSIRO Review

Chapter 3:

Close DC, Beadle CL, Holz GK and Brown PH (2001). Effect of shadecloth tree shelters on cold-induced photoinhibition, foliar anthocyanin and growth of *Eucalyptus globulus* Labill. and *E. nitens* (Deane and Maiden) Maiden seedlings during establishment. Forest Ecology and Management: (internal CSIRO review).

Chapter 5:

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Chapter 5:

Close, D.C., Beadle, C.L., Hovenden, M.J. and Brown P.H. (2001). Cold-induced photoinhibition, nutrition and growth analysis of *Eucalyptus nitens* (Deane and Maiden) Maiden seedlings during establishment. Tree Physiology: (internal CSIRO review).

Chapter 6:

Close, D.C., Beadle, C.L. and Hovenden, M.J. (2001). Sustained Xanthophyll Cycle Engagement is an overwintering strategy of *Eucalyptus nitens* (Deane and Maiden) Maiden seedlings. Oecologia: (internal CSIRO review).

Chapter 8:

Close, D.C., Beadle, C.L., Davies, N.W. and Hovenden, M.J. (2001). Effects of rapid induction and subsequent recovery from cold-induced photoinhibition on xanthophylls, anthocyanins, galloylglucoses, flavonols and sideroxylonals of *Eucalyptus nitens* seedling foliage. Plant, Cell and Environment: (internal CSIRO review).

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Chapters 5, 6 and 8:

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Technical reports

Chapters 3 and 9:

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Chapters 3, 5, 7 and 8:

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Chapters 3, 5 and 9:

Close, D.C., Holz, G.K. and Beadle, C.L. (2000). Frost vs. Photodamage of Seedlings? Beyond the Black Stump No: 22. CRC-SPF Technical Research Results Dissemination Paper.

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Definition of symbols and abbreviations

Name	Description	Units
A	Antheraxanthin	
AEST	Australian eastern standard time	
A_{max}	Light saturated net CO_2 assimilation	$\mu mol\ m^{-2}\ s^{-1}$
AMG	Australian map grid	
asl	Above sea level	
A+Z/V+A+Z	Xanthophyll-cycle conversion ratio	
CH	Artificially cold-hardened	
CID	Collision-induced disassociation	
Chl	Chlorophyll	
DAP	Diammonium phosphate	
DW	Dry weight	
Est	Established	
ETR	Electron transport rate	$\mu mol\ m^{-2}\ s^{-1}$
F	Non-shaded fertilised	
F_1, F_2 etc.	Unknown flavonol glycoside	
F_m, F_m'	Maximal chlorophyll fluorescence yield measured in dark- and light-adapted states respectively	
F_o	Minimal chlorophyll fluorescence yield measured in the dark-adapted state	
F_v/F_m	Variable to maximal chlorophyll fluorescence ratio	dimensionless
GCT	Growth chamber treatment	

Name	Description	Units
HPLC/ESI-MS	High pressure liquid chromatography interfaced to electrospray ionization mass spectrometry	
HPLC/UV	High pressure liquid chromatography interfaced to ultra violet-visible spectrophotometer	
IRGA	Infra-red gas analyser	
LAR	Leaf area ratio	$m^2\ kg^{-1}$
LHC	Light harvesting complex	
LWR	Leaf weight ratio	$kg\ kg^{-1}$
MAI	Mean annual increment	
MS	Mass spectroscopy	
MS/MS	Mass spectroscopy daughter ion production	
m/z	Molecular weight	
N	Nitrogen	% dry weight of foliage
NAR	Net assimilation rate	$g\ m^{-2}\ d^{-1}$
NF	Non-shaded fertilised	
NFP	North Forest Products	
NH	Non-cold-hardened	
NIRS	Near infra-red spectroscopy	
NL	Normalised level	
NMR	Nuclear mass resonance	
NPQ	Non-photochemical quenching	dimensionless
NS	Nutrient-starved	

Name	Description	Units
ϕ	Apparent quantum yield	dimensionless
P	phosphorus	% dry weight of foliage
PAR	Photosynthetically active radiation	$\mu mol\ m^{-2}\ s^{-1}$
PFD	Photon flux density	μmol photons $m^{-2}\ s^{-1}$
P_i	Inorganic phosphorus	
PSII	Photosystem II	
Q	Quercitin	
R	Rutin	
RGR	Relative growth rate	$kg\ kg^{-1}\ d^{-1}$
Sh-F	Shaded fertilised	
Sh-NF	Shaded non-fertilised	
SLA	Specific leaf area	
TCA	Trichloroacetic acid	
V	Violaxanthin	
V+A+Z	Total xanthophyll-cycle components	
Ψ_{pd}	Pre-dawn leaf water potential	MPa
Z	Zeaxanthin	

Chapter One. General Introduction

Rationale

The worldwide clearing of indigenous forests has resulted in some developed countries having less than 10 % of their original forests remaining. Developing countries are following this trend and largely account for the net rate of deforestation which was estimated last decade as over 5 million ha yr^{-1} (Mather 1990). This high rate of clearance and the low productivity of indigenous forests has led to increased reliance on intensively-managed plantation systems. Worldwide, if the present rate of wood consumption (3.5 billion $m^3 yr^{-1}$) was supplied by indigenous forests at a sustainable mean annual increment (MAI) of 3 $m^3 ha^{-1} yr^{-1}$, 29% of the world's remaining indigenous forests and woodlands would be required compared to 2.7% of the world's land base if this wood was sourced from intensely-managed plantations at an MAI of 10 $m^3 ha^{-1} yr^{-1}$ (South 1995).

In Australia, the forest-based industries are experiencing increasing domestic and export demand for wood products. Greater regulation of the industry has occurred through the introduction of Regional Forest Agreements and the requirement for meeting Australia's greenhouse gas emissions targets (Anon 1999). In response to these factors, the Australian government has developed a strategic plan entitled the '2020 Vision for the plantation estate in Australia' (Anon 1999). The 2020 Vision aims to treble the size of the plantation estate in Australia by the year 2020, a target that requires the planting of, on average, 80 000 ha of new plantations each year between 1997 and 2020 (Anon 1999). A reversal of the current $1.5 billion trade deficit for forest products in Australia is anticipated. In addition, the expanded forest

area could create up to 40 000 jobs in rural areas. If achieved, this will bring Australia into line with countries such as New Zealand where export of plantation wood maintains the balance of trade in wood products (South 1995).

In the current economic climate, there has been a decline in the establishment of new softwood plantations in Australia (Anon 1999). There is also, currently, a high world-wide demand for eucalypt pulp for paper products (Tibbits *et al.* 1997). A consequence is that establishing *Eucalyptus globulus* and *Eucalyptus nitens* plantations will be the major means of achieving the 2020 Vision in the commercial sector.

The cost of raising and planting seedlings is a significant proportion of the establishment costs that must be carried over 10-15 years for pulpwood regimes or 20-30 years for sawlog regimes. Death or poor performance of seedlings not suitably acclimated for a given site will increase these costs. Consequently, forest managers are seeking advice on how best to acclimate seedlings for optimal survival and performance on sites in Tasmania where low temperature is the major limitation to growth.

Background

It is generally accepted that transplant stress (Sands 1984; Rietveld 1989), planting check (South and Zwoloski 1997) or transplant shock (Kim *et al.* 1999) occurs after planting of tree seedlings where acclimation to the planting environment is required until growth is the same as naturally regenerating seedlings of the same size (Rietveld 1989). Naturally regenerating seedlings have been reported to have three times the

stem volume of nursery grown stock of the same age in the field (Bernier 1993). If the seedling is unable to acclimate it dies. Seedling mortality increases establishment costs. Re-planting may be necessary or the land resource is under-utilised. This is an expensive and time consuming exercise that can result in the harvest of under-developed trees if re-planting occurs too late.

Before seedlings establish they are inherently highly susceptible to physical and physiological stress. Lack of lignification can result in mechanical damage from wind (Telewski and Pruyn 1998), proximity to ground level increases risk of frost damage due to cold air stratification (Jordan and Smith 1995), low levels of foliar pigments and photosynthesis (Krause *et al.* 1995; Dodd *et al.* 1998) predisposes seedlings to cold-induced photoinhibition (Lundmark and Hällgren 1987; Ball *et al.* 1991; Örlander 1993; Ball 1994 and Holly *et al.* 1994) and small root systems may lead to nutrient deprivation and water stress (Stuve and Joly 1992; Greenfield and Paterson 1994; Schultheis and Dufault 1994; McGrady 1996; Wilson and Clarke 1998b; Kim *et al.* 1999). Eucalypt seedlings are also highly susceptible to browsing damage in Tasmania. The young foliage is nutritious, palatable and especially attractive to native fauna such as pademelon, possum and wallaby (Bulinski and McArthur 1999). Further, the ability of seedlings to recover from stress is limited by inherently small carbohydrate reserves (Ritchie 1982; Van den Driessche 1987; Phillipson 1988; Rietveld 1989).

It is known from experience in the forest industry that once seedlings are past the establishment phase of growth, foliar damage through abiotic factors or from vertebrate browsing is unlikely to have a major effect on survival and performance

relative to damage sustained during seedling establishment. Seedling survival and performance is a critical issue for the success of Tasmania's rapidly expanding plantation estate.

Species

E. globulus and *E. nitens* are the main hardwood plantation species used in Australia (Tibbits *et al.* 1997). These species have complementary characteristics. The natural distribution of *E. globulus* is widespread in predominantly coastal and near-coastal areas of eastern and south-eastern Tasmania, the Bass Strait islands, in the Otway Ranges and in south Gippsland in Victoria. It occurs at altitudes between 0 – 650 m, but is typically found below 400 m (Williams and Potts 1996), where the mean annual rainfall is between 500 – 2000 mm (Tibbets et al 1997). *E. globulus* exhibits rapid early growth, is relatively drought tolerant, but is intolerant of frosts. For these reasons, *E. globulus* is planted on mild sites. This species is the most frequently planted plantation species in the world (Tibbits *et al.* 1997) and accounts for most of the commercial plantings to eucalypt species in Southern and Western Australia. In contrast, *E. nitens* is relatively less tolerant of drought but is frost tolerant. Its natural distribution covers the Great Dividing Range and coastal ranges from northern New South Wales to the Victorian Alps, with most occurring around the New South Wales – Victorian Border. It occurs at altitudes between 600 – 1600 m, where the mean annual rainfall is between 750 – 1750 mm (Boland *et al.* 1994). *E. nitens* is the species of preference for cold sites. It accounts for most of the commercial plantings to eucalypt species in Tasmania and the cooler areas of Victoria.

Project justification

For many years in Tasmania, field nurseries at relatively high altitudes (*ca.* 300 m asl) have been used to produce large, cold hardy, open-rooted seedlings (Plate 1.1) for planting on frost prone sites. Knowledge, based on experience, enabled production of seedlings which performed well at high altitudes. In 1997, to minimise costs, North Forest Products Pty. Ltd.(NFP) invested in a technologically advanced, largely automated nursery at about 10 m asl which produces only containerised seedlings. The containerised seedlings are smaller than open-rooted stock and are less cold hardy (Plate 1.2) because they have little opportunity to acclimate in the warmer temperatures at sea level. Therefore there was concern about the survival and performance of the stock grown at sea level when it was planted on higher altitude, colder sites.

Plate 1.1. Large, cold hardy, open rooted seedling typical of those produced at high altitude field nurseries.

Plate 1.2. Small, non-cold hardy seedlings typical of those produced at the North Forest Products Somerset nursery at sea level.

Based on some experience in New Zealand, empirical trials undertaken by NFP around 1980 suggested that nutrient starved seedlings with 'red' leaves were able to withstand frost damage. Subsequently, these nutrient starved, red seedlings were produced routinely for frost prone sites. However, there was little quantitative data supporting this practice and the mechanisms of protection were not understood.

Summary of thesis structure

Chapter 1 forms the general introduction to this thesis. Chapter 2 describes methodologies common to more than one experimental chapter. Chapters 3-9 are experimental chapters. Chapter 10 discusses practical outcomes from this research and implications for current management practices.

Major objective

- to investigate the (photosynthetic and nutrition) physiology of seedling acclimation to cold planting conditions and its effect on seedling growth.

Following is a brief summary of the experimental chapters.

Chapter 3 describes a field trial at Watson's block (350 m asl) in north-west Tasmania. This trial was designed to identify physiological factors most vital to establishing *Eucalyptus* seedlings on cold sites. The experiment compared the physiology of *E. globulus* and *E. nitens* seedlings subjected to various pre-conditioning treatments to that of established one-year-old *E. nitens* saplings. Seedlings were planted in spring 1997.

Chapter 4 describes the nutrient physiology of seedlings after planting at Watson's Block.

Chapter 5 describes a field trial at Moory Road (800 m asl) in north-west Tasmania. In light of the results from Watson's Block four treatment combinations were used. These were assessed primarily by chlorophyll fluorescence and foliar pigment analyses. The study was done entirely on *E. nitens* planted in early winter 1998.

Chapter 6 investigates sustained xanthophyll cycle engagement. During the course of the Moory Rd trial it was observed that leaf photosystems were not relaxing during conventional half hour relaxation periods in the dark. This experiment investigates PSII efficiency and xanthophyll cycle recovery under controlled environmental conditions.

Chapter 7 identifies possible mechanisms, other than the xanthophyll cycle, that *E. nitens* employs to reduce physiological and physical damage during periods of low temperature stress, thus maximising the potential for survival and growth. Polyphenols were identified and quantified which may be antioxidants, attenuate light and deter herbivory. Polyphenol concentrations were related to cold-induced photoinhibition, leaf optical properties and invertebrate herbivory.

Chapter 8 investigates rapid foliar acclimation under controlled environmental conditions where cold-induced photoinhibition and anthocyanin production was induced. Seedling treatments were those used in the Moory Rd trial.

Chapter 9 describes a photodamage event that occurred in the North Forest Products' Somerset nursery and detrimentally affected growth of *E. globulus* seedlings.

Chapter 10 synthesises the conclusions of Chapters 3-8 and discusses the implications for nursery management of seedlings destined for planting on cold sites.

Chapter Two. General materials and methods

Environmental monitoring

Air temperature was monitored with thermocouples (0.6 mm diameter) at 10 min intervals in two locations on the trial site (Chapter 3) or within or outside tree shelters (Chapter 5) at heights of 0.15, 0.30 and 0.45 m above ground as well as at 1.3 m (reference height). PT-100 thermometers (DT-50 Datataker, Helec Sales and Systems, Hobart) were placed within and outside tree shelters (Chapter 3). Shields in 2 widths (50 and 90 mm) of white PVC plumbing pipe (with 20 mm holes drilled for ventilation) with the smaller pipe secured within the larger pipe, prevented direct contact of radiation with the thermocouples. Soil temperature, total incident shortwave radiation (Qualimetrics Inc., Q*6 net radiometer, California, U.S.A.), relative humidity (Vaisala temperature/humidity probe, Helsinki, Finland), and wind speed were also monitored at 10 min intervals (Chapters 3 and 5, results not shown). All variables were summed hourly and daily averages calculated from hourly data. Data were recorded and calculated by a CR10X measurement and datalogging system (Campbell Scientific Inc., Logan, Utah).

Chlorophyll fluorescence

All chlorophyll fluorescence measurements were made using a PAM-2000 fluorometer and 2030-B leaf-clip holder equipped with a portable PC and DA-2000 software (Heinz Walz GmbH, Effeltrich, Germany). Single measurements were made on the middle of each leaf of the most recently expanded leaf pair and on a single older leaf from the leaf pair one node back for both dark-adapted and light stable

measurements. Each measurement was replicated on three seedlings for both dark-adapted and light-stable measurements.

Dark-adapted minimal (F_o) and maximal (F_m) fluorescence were measured before dawn in Chapter 3, 5 and 8 and at specified times in Chapter 6. The saturation pulse was 0.8 s long.

Steady-state fluorescence (F) and maximal fluorescence in the light-adapted state (F_m') were measured *in situ* at various times during the day (Chapter 5, 6, 8 and 9). Actual fluorescence level, F, was monitored until it was stable. F_m' was then measured using a saturating flash of 0.8 s during exposure to natural illumination. F_m' measured at the indicated time of day in conjunction with F_m measured pre-dawn the same day were used for the calculation of NPQ. Yield was calculated as $\Delta F/Fm'$ and NPQ as $NPQ = Fm/Fm' - 1$ (Genty *et al.* 1989).

Gas exchange

Photosynthetic light-response curves were obtained for one leaf of the most recently expanded leaf pair, using an open-flow gas-analysis system (LCA2, Analytical Development Corporation, Hoddesdon, Herts, UK) (Chapters 3 and 5). A Parkinson PLC-B leaf chamber (area 6.25 cm^2) in conjunction with a lamp comprising four 150 W Wotan xenon quartz globes was used. An electric fan was incorporated into the illumination source to dissipate heat generated by the globes. Incident photon flux densities (PFDs) below 750 $\mu mol\ m^{-2}\ s^{-1}$ were obtained using neutral density filters. PFDs above 750 $\mu mol\ m^{-2}\ s^{-1}$ were obtained by increasing power to the lamp. Leaves were exposed to ascending irradiances during light response assessment. The PLC-B

leaf chamber had no temperature control, thus photosynthesis was measured under temperature of the environment at that time. Maximum net photosynthesis (A_{max}) was determined from measurement of net photosynthesis under conditions of saturating light, namely 1500 μmol m^{-2} s^{-1}. See the statistical analysis section of this chapter for detail on light response curve modeling.

Each measurement series was replicated on the same leaves of three seedlings/saplings. Measurements were made between 1100 and 1400 h AEST. Chlorophyll-fluorescence and gas-exchange measurements were made on the same day.

Sampling for pigment analysis

Pigment samples for chlorophyll and carotenoid and galloylglucose, sideroxylonal, flavonoid and anthocyanin analyses were taken within 15 minutes of chlorophyll fluorescence measurement (Chapters 5, 6, 7 and 8). Three seedlings of each treatment were selected at random and leaves of similar exposure to incident PFD selected for sampling. Leaf discs were punched, placed in pre-labelled, porous cloth bags and immersed in liquid N within 10 s. Samples remained in liquid N until transfer to a freezer held at –87 °C.

Chlorophylls and carotenoids

Extraction

Samples were transferred from the – 87 °C freezer to the laboratory on ice. Exposure to direct sunlight was prevented and the extraction was done under dim laboratory light. Leaf discs were ground in 2 ml 100% acetone with 25 mg $CaCO_3$, centrifuged

for 10 min at 5000 rpm and the resulting supernatant retained. Pellets were re-extracted as above with 1.25 ml 100% acetone (Gilmore and Yamamoto 1991). Chlorophyll and carotenoid pigment extracts were immediately frozen at –20 °C and analysed within 24 h of extraction (Chapters 5, 6 and 8).

HPLC-UV

HPLC analyses were carried out on a Waters Alliance 2690 with a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, Massachusetts) (Chapters 5, 6 and 8). The column was a Spherisorb ODS-1 (Alltech, Deerfield, IL, U.S.A.). The ODS-1 (5-μm particle size, 250 mm * 4.6 mm I.D.) is a non-endcapped, 6% carbon, spherical silica material (Phase Separations, Clwyd, U.K.). The guard column was an Alltech ODS-1 direct-connect cartridge.

The flow rate for all separations was 2 ml min^{-1}. All sample injections were 20 μl. The solvent system was; solvent A was run isocratically from 0 to 4 min followed by a 2.5-min linear gradient to 100% solvent B. Solvent mixtures were: A, acetonitrile-methonal-Tris HCl buffer 0.1 M pH 8.0 (72:8:3); B, methanol-hexane (4:1) (Gilmore and Yamomoto 1991). The columns were re-equilibrated between samples for a minimum of 10 min with solvent A. All analyses were at room temperature.

Identification and quantification

β,β-carotene was obtained from Sigma Chemical Company Pty. Ltd. (Sydney, Australia). β,β-carotene was dissolved in hexane and the extinction coefficient used for quantification was 2590 (Davies 1976). The photodiode-detector wavelength for integration of peak area was 440 nm. A conversion factor was calculated using the

absorbance peak area of β,β-carotene. Conversion factors for peak area to nmol per injection were: violaxanthin (20.72); lutein (27.10); zeaxanthin (26.90); chlorophyll *a* (34.94); chlorophyll *b* (38.53); β,β-carotene (18.94) (Gilmore and Yamamoto 1991). Antheraxanthin was estimated with the conversion factor for lutein. Neoxanthin was estimated with the conversion factor for violaxanthin (Gilmore and Yamamoto 1991) (Chapters 5, 6 and 8).

Polyphenols

Extraction

Preliminary extraction with various solvents revealed homogenisation with acidified (pH 1) 100% methanol as the most effective system. Polyphenols were extracted from three leaf discs (total area = 0.0014 m^2 for each treatment) with 2 ml acidified methanol at room temperature using a Pro-200 tissue homogeniser (Pro Scientific Inc., Monroe, CT, USA) (Chapters 7 and 8). Samples were then immersed in a water bath at 90 °C for 1.5 min and left to extract in the dark at 5 °C for 24 h (Mancinelli *et al.* 1975). Extracts were then centrifuged at 5000 rpm for 10 min. The supernatant was removed and the pellet re-extracted using the same method. Supernatants were pooled to pre-labelled containers and stored in a freezer held at –20 °C. Samples were analysed within 24 h of extraction.

Chromatographic and spectrometric analysis

HPLC/UV

HPLC analyses were carried out on a Waters Alliance 2690 with a Waters 996 Photodiode Array Detector (Chapters 7 and 8). The column was a Waters Novapak C18 column (3.9 mm x150 mm) fitted with an Alltech Econosphere 5 u C18 guard

cartridge (Alltech, Deerfield, IL, U.S.A.). The flow rate was 0.8 ml min^{-1}. The solvent system was; solvent A - 2% glacial acetic in methanol, solvent B - 2% glacial acetic in distilled water, solvent C - hexane. The solvent gradient was; 5% solvent A/95% solvent B; ramped to 35% solvent A/65% solvent B at 6 min; then to 45% solvent A/55% solvent B at 16 min; then to 100% solvent A at 25 min; then to 80% solvent A/20% solvent C at 26 min and held till 35 min. The original conditions were re-established by ramping to 100% Solvent A at 36 min and holding for 2 min, then ramping to 5% Solvent A/95% Solvent B at 40 min followed by 15 min re-equilibration. The photodiode array detector was monitored from 230 nm to 600 nm at a sampling rate of 1 spectrum s^{-1} and at a resolution of 2.4 nm.

HPLC/ESI-MS

The HPLC column and conditions were as above. The HPLC column outlet was coupled directly to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source (Finnigan MAT, ThermoQuest Co., San Jose, California, U.S.A.) (Chapters 7 and 8). The MS conditions were as follows; needle voltage 4.5 kV; capillary temperature 270 °C; capillary voltage -30 V, tube lens offset -30 V, sheath gas 90 psi, auxillary gas 50 psi. Ions from m/z 125 to 1500 were monitored, with alternate data-dependent MS/MS scans from the most intense ion, using an isolation width of 3 amu and collision energy of 30%.

Direct determination of anthocyanins by MS/MS

Direct positive ion electrospray mass spectrometry using the Finnigan LCQ confirmed the presence of strong molecular ions for ionised cyanidin-3-glycoside and cyanidin-3,5-diglycoside at *m/z* 449 and 611 (Chapters 7 and 8). The *m/z* 611 ion produced

intense daughter ions at *m/z* 449 and 287 by collisional activation, characteristic for losses of one and both glucose units (less a molecule of water), while the *m/z* 449 main beam ion also produced a daughter ion at *m/z* 287 (cyanidin) characteristic for the loss of its single glucose unit. The ratio of the main beam *m/z* 611 and 449 ions varied widely between individual samples, indicating the latter was not derived from the former.

A 'Selected Reaction Monitoring' (SRM) experiment was therefore established to look for *m/z* 449 and 287 derived from *m/z* 611, and *m/z* 287 derived from *m/z* 449 to enable targeting cyanidin-3-glycoside and cyanidin-3, 5-diglycoside, respectively, in the crude sample. The parent ions were isolated with a window of 4 amu, and the collision energy was 25%. Daughters from *m/z* 282 - 292 were monitored for cyanidin-3-glycoside, and daughters from *m/z* 282-292 and 444 - 454 were monitored for cyanidin-3,5-diglycoside using peak profile mode. The samples were introduced as 10 μL injections into a flow of 200 μL min^{-1} of 5% acetic acid connected directly to the electrospray source. The electrospray needle voltage was 5.4 kV, the sheath gas 65 psi, capillary voltage 20 V and the tube lens offset 40 V. For quantification the range *m/z* 286-288 (from 449) was integrated for cyanidin-3-glycoside, and *m/z* 448 - 450 (from 611) for cyanidin-3,5-diglycoside.

Anthocyanins were extracted from anthers of *Callistemon* species (Cornford 1993) and a concentrated source of cyanidin-3-glycoside isolated. This produced an identical MS/MS spectrum to that identified as cyanidin-3-glycoside in *E. nitens* extracts.

MS/MS anthocyanin levels were quantified by application of a correction factor calculated from absorbance in a UV-visible spectrophotometer (Varian Carey 1E, Australia) at 528 nm. This was corrected for chlorophyll absorption by subtracting $0.24A_{653}$ (Mancinelli and Rabino 1984) using a molar extinction coefficient of 30000 (Murray and Hackett 1991).

Identification of compounds

Galloylglucose and sideroxylonal

Previous work on eucalypt wood extracts (Barry *et al.* In press) established that HPLC/ESI-MS with negative ion detection, in conjunction with collision-induced disassociation (CID) MS/MS daughter ion production, was a suitable method for identification of individual tannins to at least molecular weight and pattern of general substitution. A range of specific galloylglucose standards was analysed and general rules for assignment of other galloylglucoses established. Penta-galloylglucose and other positional isomers (di-, tri-, and tetra-galloylglucose) were identified as the major contributors to the foliar tannin fraction. A strong correlation was found between the UV chromatogram at 280 nm and the total ion chromatogram (TIC) of the galloylglucoses and sideroxylonals. This was supported by MS/MS daughter ions spectra from the $[M-H]^-$ ions. The presence of sideroxylonal A and B was confirmed by comparison of HPLC, UV and MS data with standards.

Flavonoid

Flavonoids were identified initially by characteristic UV spectra measured at 370 nm. The specific flavonoids quercetin and rutin (quercetin 3-O-rutinoside) were confirmed by comparison of HPLC, UV and MS data with standards. Assignments of other

flavonoids as quercetin glycosides were based on appearance of characteristic $[M-H]^-$ ions at *m/z* 463 together with MS/MS spectra characterised by loss of the sugar group to yield ions at *m/z* 301 and 300.

Anthocyanin

The dominant anthocyanins in eucalypts in the *Macrantherae* are cyanidin-3-glycoside and cyanidin-3,5-diglycoside (Sharma and Crowden, 1974). MS data indicated detectable amounts of both these cyanidin species in extracts of *E. nitens* foliage. Levels of both cyanidins were pooled for the purpose of temporal comparison.

Semi-quantification of sideroxylonals, galloylglucoses, flavonoids and anthocyanins

Investigation of UV data obtained at 280 nm (at < 10 min and ~28 min for galloylglucoses and sideroxylonals respectively) and 370 nm revealed that minimal absorption occurred at these wavelengths other than by galloylglucoses, sideroxylonals and flavonoids respectively. Total absorption at these wavelengths was therefore pooled and used for semi-quantitative evaluation (Chapters 7 and 8). Correction factors calculated from tetra-galloylglucose, sideroxylonal A and B, and quercetin standards were applied for the quantification of galloylglucoses, sideroxylonals and flavonoids respectively.

Visible/near infra red reflectance spectroscopy (VIS-NIRS)

The two most recently expanded leaves of 10 randomly selected seedlings of each treatment were excised and immediately placed in petri dishes containing moistened paper towelling and stored at 4 ± 1 °C. Leaves maintained in this state were measured by Near Infra Red Spectroscopy (VIS-NIRS) within 48 h of excision (Chapters 5 and 8).

Leaf reflectance was measured with a spectrophotometer (NIRSystems Model 6500, Perstorp NIRSystems Inc., Lindbrook International-Foss Pacific, Silver Spring, Maryland, U.S.A.). Two replicate measurements (of 64 scans) of monochromatic light were made at 2-nm intervals over a range from 400 to 800 nm to produce an average spectrum of 1050 data points. The bandwidth was 10 nm and the wavelength accuracy ± 0.5 nm. Wavelengths were calibrated by reference to didymium and polystyrene standards. Reflected radiance was measured at 45° angle above and below the incident beam which was normal to the target. Reflectance (R) was converted to absorbance (A) using the equation: $A = \log (1/R)$. Reflectance was measured on the adaxial surface of leaves (with midveins removed) covering a 45 x 200 mm^2 reflectance cell.

Growth analysis

Seedling height data were separated into four height classes and ten seedlings representative of each of the classes selected for harvesting. Soil was washed from the root systems and total single-sided leaf area measured. Leaf, stem, branch and roots were oven dried at 70 °C prior to measurement of dry mass. Growth analysis variables were calculated according to Beadle (1993).

Nutrient analysis

Total N and P

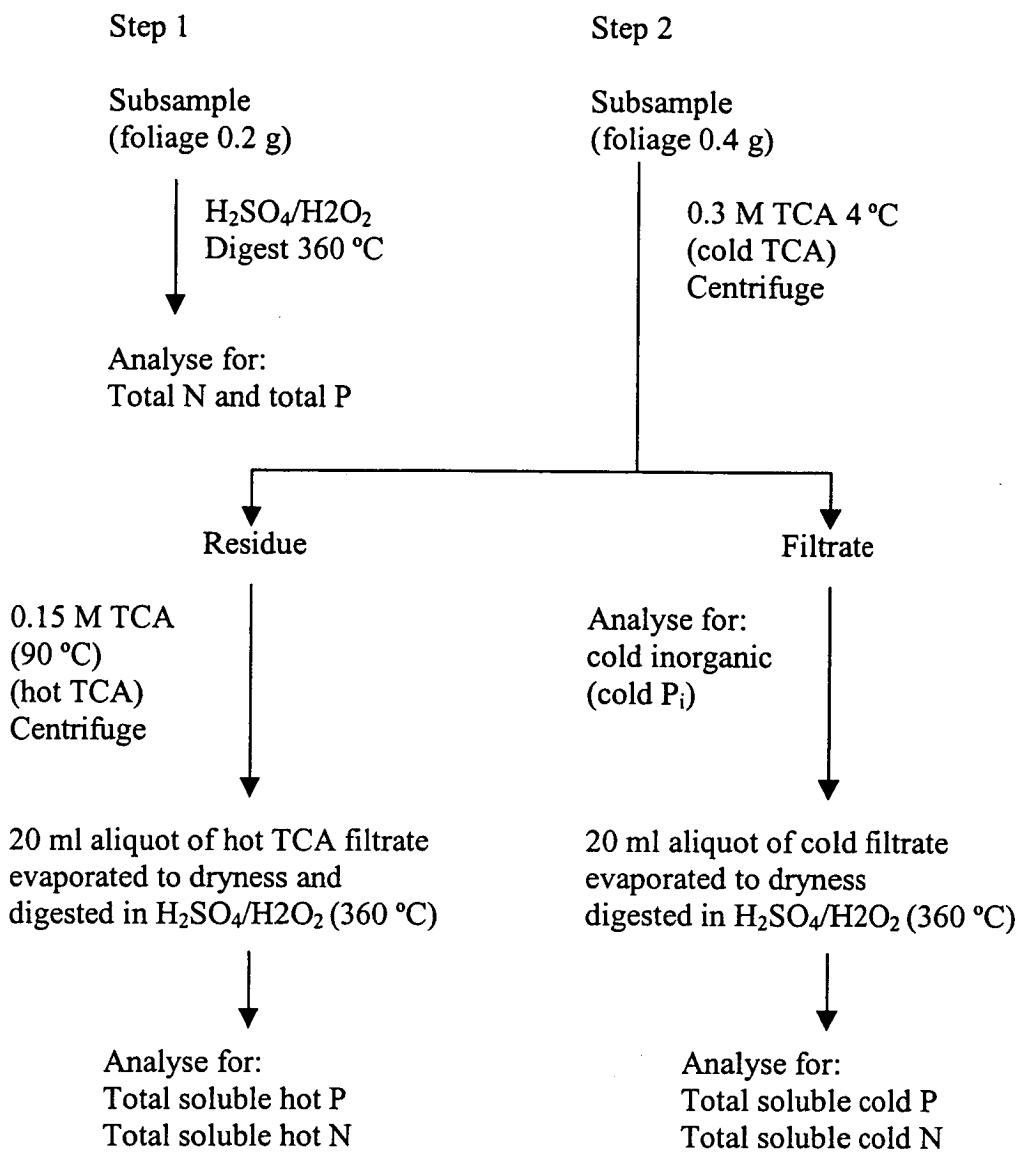
For nutrient analyses, foliage from (10) seedlings used for growth analyses was bulked into three samples (Chapters 3 and 5). Twelve seedlings each were destructively sampled and bulked before and after growth chamber experiments (Chapter 8). Foliage from each bulked sample was used for three (Chapters 3 and 5) or two (Chapter 8) replicates of total and TCA nutrient analyses. Plant material was dried at 70 °C to constant weight and then ground in a hammer mill. Approximately 0.16 g of re-dried, ground material was digested in 4 mL of concentrated H_2SO_4 and 2 mL of H_2O_2 (30% w/v) at 360°C for 30 minutes (Lowther 1980). After cooling to 150 °C, hydrogen peroxide was added dropwise until the solution cleared to a pale yellow colour. The samples were then digested at 360 °C for a further 60 minutes, resulting in a clear digest. The digest was diluted, and colourimetrically analysed for N (QuikChem method 10-107-06-2E, Lachat Instruments, Wisconsin, U.S.A.) and P (QuikChem method 10-115-01-1d, Lachat Instruments) on a continuous flow injection analyser (QuikChem 800, Lachat Instruments) (McLeod 1992). Standard samples (± 10 %, source: Australasian Soil and Plant Analysis [ASPAC]) of known N and P concentration and blank samples were included to validate the efficiency of digestion and elemental analysis.

Trichloroacetic acid (TCA) analysis for N and P chemical fractions

The trichloroacetic acid (TCA) fractionation method of Kedrowski (1983) as modified by Polglase *et al.* (1992a) was used to extract the various N and P fractions from foliage samples. Oven-dried samples (0.4 g) were extracted initially in 50 mL of 0.3

M TCA at 4 °C for 1 h, with shaking every 10 minutes. This was followed by centrifuging, supernatant removal and re-extraction of the residue in 0.15 M TCA at 90 °C for 1 h. A sub-sample (20 ml) of the supernatant from each of the cold and hot extraction procedures was evaporated to dryness at 100 °C and digested for total N and P determination as described above. The method of producing extracts and the notation used to describe these extracts is shown in Figure 2.1.

The fractionation of N and P in foliage and litter samples is presented according to Kedrowski's (1983) classification. To summarise the fractions, more labile inorganic forms are extracted in TCA at 4 °C (NH_4^+ and HPO_4^{2-}) while relatively less labile fractions are extracted in TCA at 90 °C (phytate-P and nucleic acid related N and P). The residual N and P following TCA extraction comprises relatively insoluble P complexes and protein N (Polglase *et al.* 1992a). Inorganic N was initially found to be an insignificant component of the total N pool (similarly reported by Polglase *et al.* 1992a) and was omitted from further analysis.



Extract notation

Total N/P = total digest in H_2SO_4/H_2O_2 N/P (step 1)
Inorganic P = P extracted in 0.3 M cold TCA
Soluble (nitrate, ammonia and amino acid) N/sugar phosphate P = total soluble cold N/P
Nucleic acid N/P = N/P extracted in hot TCA 0.15 M and digested in H_2SO_4/H_2O_2
Protein N/insoluble P complexes = step 1 minus other N/P fractions

Figure 2.1. The sequence of extraction of foliage samples with trichloroacetic acid (TCA) and the notation of fractions derived (from Hooda and Weston 1999).

Extraction notation for N is derived similarly to that of P.

Statistical analysis

Treatment effects on reported variables were tested using one-way analysis of variance and the ANOVA procedure of SAS (SAS Institute Inc. 1989). Where one-way analyses were used to compare measurements between dates there is a higher chance of Type 1 errors occuring compared to a two-way analysis of variance. Given the very low probablilities observed, the reported differences probably exist, however re-analysis of data using two-way analysis of variance will be conducted before publication of data.

A non-rectangular hyperbolic function was used to describe the light response curves of seedlings (Pinkard *et al.* 1999):

$$A = 2\alpha Q A_\chi / (\alpha Q + \text{sqrt}[(\alpha Q + A_\chi)^2 - 4\theta Q A_\chi]) - r$$

where α approximates the efficiency of photosynthesis (quantum yield ϕ); A_χ gives the light-saturated value of A (A_{max}); θ describes the slope of the curve; and A and Q are the assimilation rate (A) and PFD, respectively and r is an estimate of respiration (Long and Hällgren 1993). Where data are fitted to straight line models, linear regression analysis (PROC GLM in SAS) was used to compare the stated variables. In all analyses the least squares method was used to estimate means and standard errors.

Chapter 3. Effects of hardening pre-treatments after an early-spring planting at 350 m asl

Introduction

What is photoinhibition?

Sustained decreases in the efficiency with which photosystem II converts the energy of absorbed photons into electron transfer occur in leaves exposed to high levels of irradiance and low temperature (Adams *et al.* 1994). Low temperature also limits the rate at which Calvin cycle enzymes function (Powles 1984). These changes are manifest in a reduced photosynthetic capacity (Berry and Björkman 1980). If light is absorbed in excess under these environmental conditions, the excess energy must be dissipated or the chloroplast membranes sustain oxidative damage (Foyer *et al.* 1994). Photoinhibition of photosynthesis can prevent such damage (Huner *et al.* 1993). Dynamic photoinhibition is a rapidly reversible process involving photoprotective mechanisms such as the xanthophyll cycle (Demmig-Adams and Adams 1992) which decrease the efficiency of photosynthesis by dissipating excitation energy as heat. Chronic photoinhibition is a slowly reversible process involving destruction or inactivation of PS II (Osmond 1994) which decreases photosynthetic capacity as well as efficiency.

Where photoinhibition occurs

Evergreen species that grow in cold climates frequently experience high irradiance and low temperature. Cold-induced photoinhibition has been observed in natural environments at high altitudes (Leverenz and Öquist 1987; Öquist and Huner 1991; Ottander and Öquist 1991; Verhoeven *et al.* 1996; 1999); high altitude eucalypts (Ball

et al. 1991; Holly *et al.* 1994; Ball *et al.* 1997); seedlings of *Eucalyptus globulus* Labill. growing in a nursery (Close *et al.* 1999a); seedlings of *E. globulus* and *Eucalyptus nitens* (Deane and Maiden) Maiden established in plantations (Close *et al.* 1999b); as well as annual crops such as *Brassica napus* L. (Farage and Long 1991) and *Gossypium hirsutum* L. (Königer and Winter 1991).

Effects of shading on regeneration

The establishment and distribution of regenerating tree seedlings has been shown to reflect the degree of cold-induced photoinhibiton. For example, high levels of incident irradiance restrict *Picea englemannii* Parry ex. Engelm. and *Abies lasiocarpa* (Hook.) Nutt. (Germino and Smith 1999) and *Eucalyptus pauciflora* Sieb ex Spreng (Ball *et al.* 1991) to particular microsites near their low temperature limits of distribution. *E. pauciflora* regenerates under the south-western aspect of established tree canopies where they are protected from exposure to high light and night-time radiation frosts (Ball *et al.* 1991). Natural or artificial shading of regenerating seedlings has been shown to alleviate effects of cold-induced photoinhibition (Lundmark and Hällgren 1987; Oberhuber and Bauer 1991; Örlander 1993; Holly *et al.* 1994). Shadecloth tree shelters minimised cold-induced photoinhibition and maximised growth of *Eucalyptus polyanthemos* Schau. seedlings compared to exposed seedlings on cold, open pasture sites (Holly *et al.* 1994).

E. nitens ***seedlings are pre-disposed to photoinhibition***

In Tasmania, large areas of eucalypts are planted at high altitudes with low mean annual temperatures (<10 °C). High pressure systems in winter bring clear night skies and the leaf temperature of seedlings can drop below air temperature due to radiative

cooling (Jordan and Smith 1995). Cold air stratification near ground level further increases the exposure of the leaves to low temperature (Jordan and Smith 1994) ahead of photoinhibitory conditions (low temperature/high light) developing early in the morning. *E. nitens* is the preferred species for planting in these cold environments to which *E. globulus* is poorly adapted (Tibbits 1986). The seedlings are produced in nurseries at low altitudes. In nursery trays, seedlings self-shade to a large degree. Thus seedlings are not only unused to conditions of low temperature at planting, they are also not acclimated to conditions of high light. Damage to seedlings of a range of coniferous species has been reported to result from sudden increases in incident light conditions, the degree of damage being species-specific (Gnojek 1992; Leiffers *et al.* 1993; Spunda *et al.* 1993; Mohammed and Parker 1999). Adams *et al.* (1994) hypothesised that this is due to differences in the capacity of the xanthophyll cycle to dissipate energy. Inherently low levels of photosynthesis also pre-dispose immature leaves to cold-induced photoinhibition (Krause *et al.* 1995; Dodd *et al.* 1998).

Seedling hardening

A nursery practice used to harden seedlings prior to transplanting is nutrient starvation (Close *et al.* 1999b). This has been shown to maximise survival and growth after planting and to provide a degree of acclimation to low temperature. The treatment induces the synthesis of large amounts of anthocyanins in the leaves (Close *et al.* 1999b) although the physiological function of these pigments is unclear at present (Barker *et al.* 1997; Dodd *et al.* 1998). Cold-hardened *E. nitens* seedlings are also less susceptible to cold-induced photoinhibition than non-hardened seedlings (Warren *et al.* 1998). Until recently, the importance of cold-induced photoinhibition during seedling establishment was not recognised and nursery practice was focused on

assessment of frost tolerance (Raymond *et al.* 1986; 1992a; 1992b). Impaired performance and mortality of newly planted seedlings on cold sites was considered to be caused by frost alone or on some sites, by water stress. The latter phenomenon has been reported for field-nursery grown *E. nitens* seedlings which lose considerable root mass during harvesting prior to planting (Wilson and Clark 1998a, b). Water stress can be a major factor in successful establishment of containerised tree and vegetable seedlings, even in moist environments, due to high water content per unit fresh weight (Struve and Joly 1992; Greenfield and Paterson 1994; Schultheis and Dufault 1994; McGrady 1996) and/or poor root ball to soil contact (Burdett and Brand 1990; Wilson and Clark 1998b).

Hypotheses tested

In the experiment described in this chapter, seven hypotheses are proposed and then tested;

- that recently planted seedlings will exhibit higher levels of water stress relative to established one-year-old *E. nitens* saplings;
- that recently planted seedlings will sustain frost damage to a greater degree than established one-year-old *E. nitens* saplings;
- that established, fully acclimated one-year-old *E. nitens* saplings will experience less photoinhibition than recently planted *E. nitens* seedlings;
- that artificially cold-hardened *E. nitens* and nutrient-starved *E. nitens* seedlings will experience less photoinhibition than non cold-hardened *E. nitens* seedlings;
- that non-hardened *E. nitens* seedlings will experience less photoinhibition than non cold-hardened *E. globulus* seedlings;

• that shaded seedlings will experience less photoinhibition than non-shaded seedlings and;

• that the degree of photoinhibition measured in seedlings will be reflected in reduction of seedling growth.

Materials and methods

Site description

A 1 ha trial was established on an ex-pasture site (Watson's Block) approximately 15 km south of Ridgley (397150 E 544250 N AMG reference) in Tasmania at an altitude of 350 m asl. One section of the trial (0.25 ha) was planted in 1996 and a second section in 1997 (0.75 ha). The latter area was treated with 2.5 l ha^{-1} glyphosate (Roundup, 450 $g.l^{-1}$ active ingredient [a.i.]) and 0.6 l ha^{-1} metsulfuron methyl (Brushoff, 600 $g.kg^{-1}$ a.i.) in June 1996. The former area was treated with glyphosate only. Bare soil was mounded using a plough approximately 2 months prior to planting. The mounds were cultivated with a rotary hoe to create a fine tilth and allow maximum contact between potting medium and soil. Soil type was lithic eutrudox (or brown ferrosol) with an average soil depth of approximately 50 cm. Minimal weed growth ensured mounds remained bare during the 23-week experimental period. A weather station located 0.5 km west of the site (also at 350 m asl) indicated that mean annual rainfall and temperature were approximately 1390 mm and 9.7 °C, respectively.

Plant material

Seedlings were raised from single family seedlots 2078, 2202 and 2972 *E. nitens* and seedlot 2200 *E. globulus* (ex. North Forest Products [NFP] Pty Ltd, Burnie,

Tasmania). 2078 and 2200 seedlots were germinated in the first week of March 1997 and grown for approximately one month in a nursery (Hills Pty Ltd, Devonport, Tasmania) before transportation to Hobart. At the nursery, seedlings were fertilised every 10 days with Aquasol (1g l^{-1}). A number of the 2078 and 2200 seedlings were raised at the Hills nursery until the beginning of the experiment (Table 3.1).

In Hobart, one half of the 2078 *E. nitens* seedlings were cold-hardened (referred to as CH *E. nitens*) for 2.5 months from July 1 until September 15. Each day during this period seedlings were placed in a cool room at 2 °C for 14 h overnight and moved outside at approximately 08:00 h Australian Eastern Standard Time (AEST) every morning. The other seedlings (non-hardened *E. nitens* [NH *E. nitens*] and NH *E. globulus*) were grown under the prevailing diurnal conditions in Hobart (Table 3.1). During this 2.5 month period CH and NH *E. nitens* and NH *E. globulus* were irrigated with Hoagland's solution (7.5 g l^{-1}) at 4 week intervals and immediately prior to planting. Seedlings raised entirely at the Hills nursery were similarly fed Aquasol until the commencement of the experiment.

Seedlots 2202 and 2972 were germinated in March 1996 and raised at the NFP nursery under ambient environmental conditions (Table 3.1). Seedlings were fertilised every 10 days with Aquasol (1g l^{-1}) until attaining their planting height. Subsequently, the 2972 seedlings were nutrient starved in the nursery (referred to as NS *E. nitens*) for approximately one year. The 2002 seedlings (referred to as established *E. nitens* saplings) were planted in October 1996 and received 100 g diammonium phosphate (DAP) fertiliser near the root collar 2 months post-planting.

Table 3.1. Seedling seedlot, height at planting and mean temperatures during the pre-experimental period. The treatments were CH (cold-hardened), NH (non-hardened) and NS (nutrient-starved) (planted in 1997) and established (planted in 1996).

Treatment	*E. nitens* CH	*E. nitens* NH	*E. nitens* NS	*E. globulus* NH	*E. nitens* Hills[1]	*E. globulus* Hills[1]	*E. nitens* Est.
Seedlot	2078	2078	2972	2200	2078	2200	2202
Height (cm)	9.2	10.5	15.2	16	15.9	23.5	65.2 [2]
Mean daily min (°C)	2	5	2.5	5	3.5	3.5	2.4 [3]
Mean daily max (°C)	12	12	9.5	12	14	14	9.5 [3]

[1] The Hills Nursery is located 3 km west of Devonport, Tasmania. It is less than 1 km inland from the coast at approximately 20 m asl on a north facing slope.

[2] Heights at planting of 1997 seedlings.

[3] Mean daily minimum and maximum temperatures at the experimental site during August - September 1997.

Experimental design

The established *E. nitens* saplings were planted in the 0.25 ha area (Figure 3.1) with between- and within-row spacings of 3.5 m and 2.5 m, respectively. Nine adjacent saplings were removed from a row within this planting in August 1997. The cleared section of mound was recultivated with a rotary hoe. New seedlings were planted in September 1997. They were arranged in groups of three consisting of one each of CH and NH *E. nitens* and NH *E. globulus*. A randomly selected seedling was planted in

the cleared mounds, opposite an established *E. nitens* sapling in the adjacent row. The other two seedlings in the group were planted 0.6 m either side (in the direction of the row) of the central seedling. Thus there were 9 groups of three seedlings from each treatment. These seedlings and the adjacent saplings were used for physiological measurements (Figure 3.1).

In the 0.75 ha area, CH and NH *E. nitens* and NH *E. globulus* were planted also in September 1997 in the same arrangement (Figure 3.1). Spacing between rows was also 3.5 m. These seedlings were planted into three blocks perpendicular to the incline on the site and used for growth analysis and height measurements. Each block consisted of three rows, each row containing 20 seedling groups. NS *E. nitens* seedlings with two other nursery treatments (not reported here) were planted similarly in separate groups of three seedlings in an unreplicated block (Figure 3.1). This block consisted of four rows, each row containing 10 seedling groups. Shaded and non-shaded seedlings were planted also in an unreplicated block adjacent to the NS *E. nitens* seedling block (Figure 3.1). This block consisted of four rows, each row containing three seedling groups. Seedling groups of four, consisting of one each of shaded *E. globulus*, non-shaded *E. globulus*, shaded *E. nitens* and non-shaded *E. nitens* (referred to as Sh *E. globulus*, N-Sh *E. globulus*, Sh *E. nitens* and N-Sh *E. nitens*, respectively) were planted. *E. nitens* and *E. globulus* seedlings used in this block were the 2078 and 2202 seedlings raised at the Hills nursery prior to the experiment (Table 3.1). Both unreplicated blocks had between- and-within row spacings of 3.5 and 0.6 m respectively. 50% shadecloth tree shelters supported by three wooden stakes were erected around seedlings allocated for shaded treatments immediately after planting. The open-topped shadecloth tree shelters were found to

have no effect on overnight or early morning temperatures (Appendix 1) or light quality (Appendix 2) experienced by seedlings. Fourteen weeks after planting all shadecloth tree shelters were removed. Buffer seedlings were planted with between and within row spacings of 3.5 m and 2.5 m, respectively, between experimental blocks on the 0.75 ha area (Figure 3.1). No fertilisers, herbicides or insecticides were applied throughout the period of the experiment.

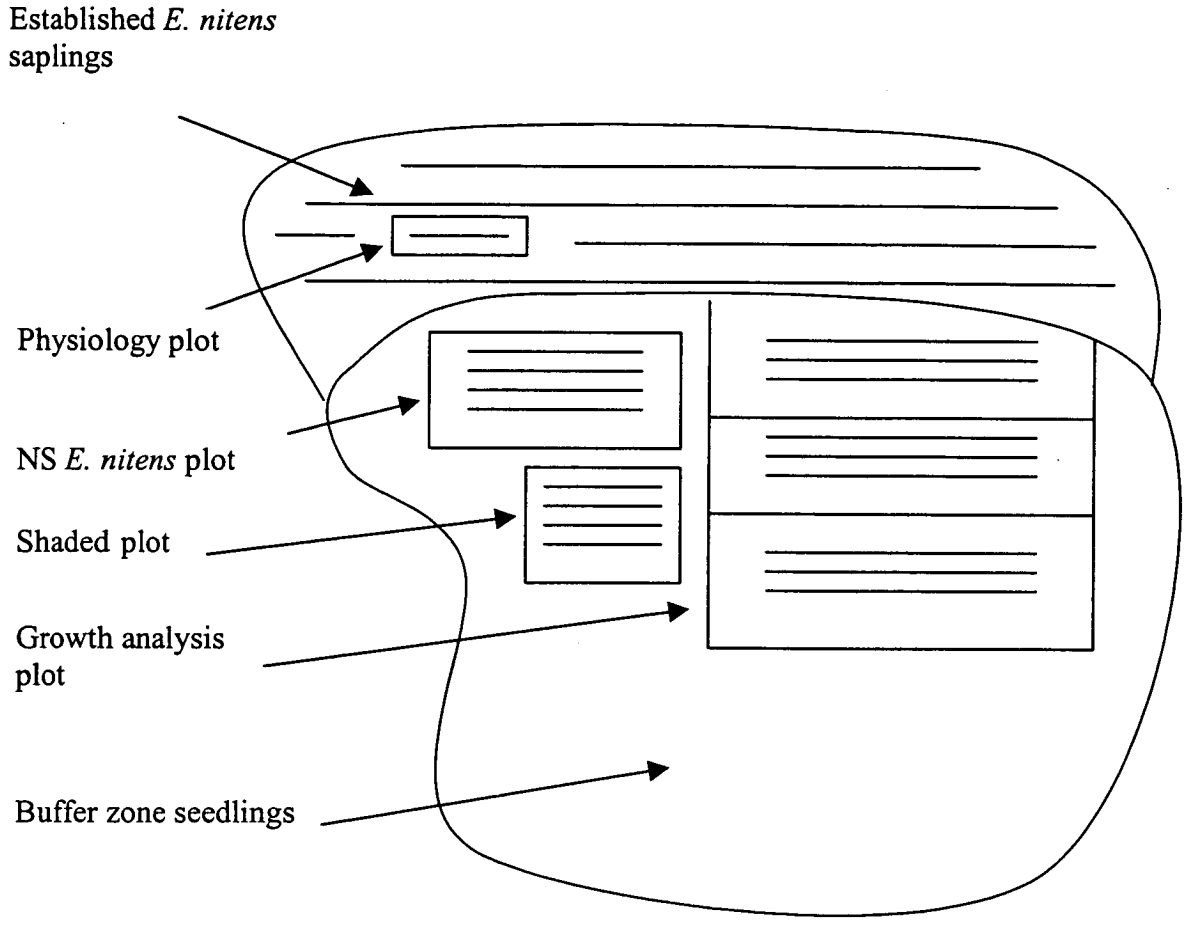


Figure 3.1. Watson's Block experimental layout showing the 0.25 ha area of established *E. nitens* saplings and the 0.75 ha area planted to seedling treatments. Within these areas, the relative position and number of seedling rows within the physiology, NS *E. nitens*, shaded and growth analysis plots are indicated.

Environment

Environmental variables were measured and recorded as described in Chapter 2.

Plant Measurements

Pre-dawn water potential

Pre-dawn leaf water potential (ψ_{pd}) was measured on the most recently expanded leaf pairs. Measurements were made between 0400 and 0600 h AEST on leaves of three randomly selected seedlings of each treatment using a pressure chamber (Model 1002, PMS Instrument Co., Corvallis, Oregon).

Chlorophyll fluorescence and gas exchange

The procedures used to measure pre-dawn maximal photochemical efficiency, F_v/F_m and gas exchange were detailed in Chapter 2. F_v/F_m of all treatments was measured. Light response of gas exchange of all treatments except Sh *E. nitens*, Sh *E. globulus* and NS *E. nitens* was measured. Measurements were made on 15 September, 8 and 21 October and 24 November 1997 and 6 January, 4 February and 5 March 1998 (0, 4, 5, 10, 16, 20 and 24 weeks after planting respectively).

Height

Measurements of heights were made on the 20 seedling groups in the middle row of each block containing CH and NH *E. nitens* and NH *E. globulus* seedlings, and of all NS *E. nitens* and Sh and N-Sh *E. globulus* and *E. nitens* seedlings. Seedling leaf necrotic area (caused by photodamage) and mortality were estimated visually during seedling height measurement. Leaf damage was ranked as nil, moderate or severe. Height measurements were made on 14 September, 22 October, 18 November and 10 December 1997 and 6 January, 4 February and 5 March 1998 (0, 5, 9, 12, 16, 20 and 24 weeks after planting respectively).

Growth analysis

Seedlings of CH and NH *E. nitens* and NH *E. globulus* only were harvested for growth analysis from the outer two rows of the blocks containing these treatments (Figure 3.1) on 14 September, 18 November, 10 December 1997, 4 February and 12 August 1998 (0, 9, 12, 20 and 47 weeks after planting).

Laboratory measurements

Pigment analysis

The most recently expanded leaf pairs of three randomly selected seedlings of NH *E. globulus*, CH and NS *E. nitens* and established *E. nitens* saplings were sampled on 15 September, 23 October, 19 November and 10 December (0, 5, 9 and 12 weeks after planting). Leaves were immediately shielded from light and stored in a cool box (4 ± 1 °C) prior to freezing at -20 °C within 24 h.

A representative sample of leaf tissue (0.2 g) was cut and ground in liquid nitrogen using a mortar and pestle. Chlorophyll and carotenoids were extracted in 2 ml 80% aqueous acetone buffered to pH 7.8 with sodium phosphate. The homogenate was collected and combined with three 1.5 ml washings of the mortar and pestle, vortexed and centrifuged at 2500 rpm for 10 min (Porra *et al.* 1989). The resulting supernatant was diluted four-fold. Absorbance was measured using a UV-Vis spectrophotometer (Varian Carey 1E, Sydney, Australia) at 663 and 646 nm for chlorophyll determination (Porra *et al.* 1989). Absorbance at 470 nm was measured for carotenoid determination (Lichtenthaler 1987).

Anthocyanins were extracted using the same volumes as above with 100% ethanol acidified to pH 1 with conc. HCl. The homogenate was then immersed in boiling water for 1.5 min and left to extract for 24 h in the dark at 5 °C. Extracts were centrifuged as above before absorbance measurements at 530 and 657 nm. The formula A_{530} - 0.25 x A_{657} was used to correct for chlorophyll and degradation products (Mancinelli *et al.* 1975). Pigment analyses of all treatments except shaded and non-shaded *E. nitens* and *E. globulus* were conducted.

Artificial frosting

Leaf discs, 8 mm in diameter, were cut with a punch from the most recently expanded leaf pair of three seedlings/saplings and placed in test tubes. Racks of test tubes were placed in baths containing 30% aqueous ethylene glycol solution and chilled at 4 °C h^{-1}. Electrolyte conductivity of 2 ml deionised water containing leaf discs was measured just before planting and at week 7 after planting, using chilling temperatures of -4 °C and -7 °C. At week 17, chilling temperatures of -2 °C and -4 °C were used. Frost sensitivity index was expressed as a ratio of electrolyte conductivity measured after cooling treatment relative to maximum electrolyte conductivity measured after immersion of discs in a 70 °C waterbath (Raymond *et al.* 1986). Frost sensitivity was assessed in all treatments except Sh and N-Sh *E. nitens* and *E. globulus*.

Statistical analysis

There were no statistically significant effects of block on mean seedling heights. At each sampling date, treatment effects on chlorophyll fluorescence, frost tolerance index, total chlorophylls, carotenoids and anthocyanins were tested using one-way analysis of variance and the ANOVA procedure of SAS (SAS Institute Inc. 1989) for

one way ANOVA. A non-rectangular hyperbolic function was used to describe the light response curves of seedlings (Pinkard *et al.* 1999):

$A = 2\alpha Q A_\chi / (\alpha Q + \text{sqrt}[(\alpha Q + A_\chi)^2 - 4\theta Q A_\chi]) - r$

where α approximates the efficiency of photosynthesis (quantum yield ϕ); A_χ gives the light-saturated value of A (A_{max}); θ describes the slope of the curve; and A and Q are the assimilation rate (A) and PFD, respectively and r is an estimate of respiration (Long and Hällgren 1993). Analysis of variance (PROC ANOVA in SAS) was used to quantify treatment effects on ϕ and A_{max}. Linear regression analysis (PROC GLM in SAS) was used to compare changes in ϕ, α and A_{max} with time as these variables increased linearly with time after planting.

Results

Environment

Differences in minimum temperatures measured at 0.15, 0.30, 0.45 and 1.3 m above ground were always within 0.5 °C. There were six frost events (with the lowest at -3.3 °C) within 3 weeks of planting. No frosts occurred after this time. During the period of measurement minimum and maximum temperatures rose steadily (Table 3.2).

Table 3.2. Number of frosts and absolute and average minimum and maximum air temperatures (°C) measured at 0.3 m in the weeks preceding each set of physiological measurements in the field. Measurements commenced on 15/9/1997.

Week	Frosts	Air Temperature			
		Minimum		Maximum	
		Absolute	Average	Absolute	Average
1	1	-0.4	3.7	16.6	14.6
3	5	-3.3	2.1	16.8	14.3
5	0	1.9	3.9	18.8	13.9
9	0	0.4	5.6	22.2	17.5
15	0	1.2	6.2	23.8	17.6
19	0	2.9	8.7	28.1	22.8
23	0	4.3	6.9	28.4	24.6

Artificial frosting

Before planting, NH *E. globulus* seedlings were least frost tolerant ($p < 0.001$), established *E. nitens* saplings and CH *E. nitens* seedlings were most frost tolerant ($p < 0.05$) and NH *E. nitens* and NS *E. nitens* had intermediate frost tolerance (Figure 3.2).

A significant dehardening had occurred by week 7 after planting in established *E. nitens* saplings ($p < 0.01$), CH *E. nitens* ($p < 0.001$), and NH *E. nitens* ($p < 0.001$) seedlings. By comparison NH *E. globulus* ($p < 0.01$) and NS ($p < 0.01$) *E. nitens* seedlings had a significantly increased frost tolerance index. Comparison of treatments at week 7 showed NS *E. nitens* and NH *E. globulus* seedlings had greater frost tolerance compared to other treatments. There were no significant differences in frost tolerance between treatments at week 17 (Figure 3.2).

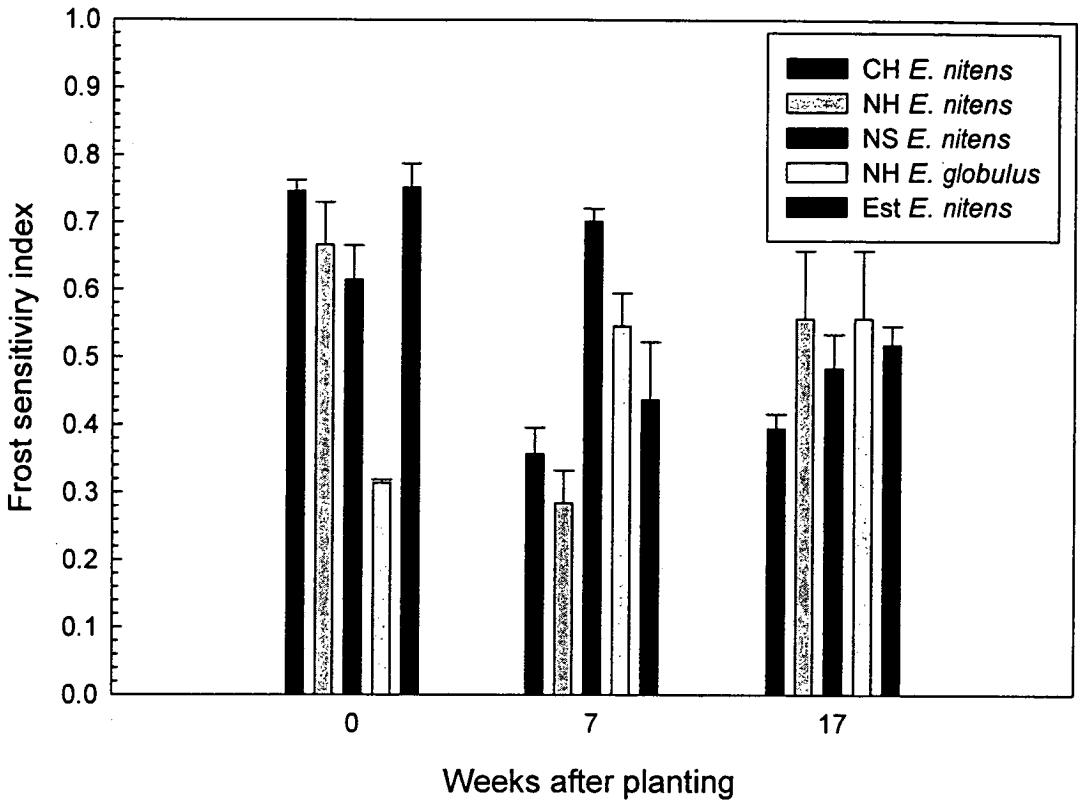


Figure 3.2. Frost sensitivity index (= electrolyte conductiviy after frosting / absolute electrolyte conductiviy) of four seedling treatments and established *E. nitens* saplings (Est *E. nitens*) at 0, 7 and 17 weeks after planting. At weeks 0 and 7, frost tolerance was assessed at the test temperature of –7 °C. At week 17, the test temperature was –4 °C. Bars indicate one standard error.

Leaf water potential

Pre-dawn water potentials were > -0.5 MPa and remained similar between treatments throughout the experimental period (results not shown).

Visually estimated photodamage

Five weeks after planting no photodamage was observed in NS *E. nitens* (Plate 3.1), Est *E. nitens* saplings (not shown), Sh *E. nitens* (Plate 3.4) or *E. globulus* (Plate 3.2). Damge was ranked as moderate in NSh *E. nitens* (Plate 3.3) and as severe in NSh *E. globulus* (Plate 3.2).

Plate 3.1. NS *E. nitens* seedlings 5 weeks after planting.

Plate 3.2. Sh (left) and NSh (right) *E. globulus* seedlings 5 weeks after planting.

Plates 3.3 and 3.4. Shaded and non-shaded *E. nitens* seedlings, respectively, 5 weeks after planting.

Chlorophyll fluorescence

F_v/F_m of NH *E. globulus* seedlings decreased markedly following planting (Figure 3.3a). The lowest levels (< 0.3) were recorded at weeks 4 and 5 and were associated with severe damage, leaf abscission and a moderate level (20%) of seedling mortality. Young, expanding, less hardy leaves had senesced by week 5. F_v/F_m of residual mature leaves and new expanding leaves had relatively constant and high values (> 0.7) at week 10.

F_v/F_m of CH and NH *E. nitens* seedlings decreased until week 5 and this was associated with moderate leaf damage. However, F_v/F_m increased to maximal values at week 9. During this period NH *E. nitens* appeared to have lower F_v/F_m levels than CH *E. nitens* seedlings but differences were not significant. F_v/F_m of both these treatments was significantly lower ($p < 0.001$) than that of established *E. nitens* saplings between weeks 1 and 9. F_v/F_m of *E. nitens* saplings was relatively constant and high ($F_v/F_m > 0.7$) throughout the experiment and no damage was observed.

F_v/F_m levels of NS *E. nitens* seedlings increased steadily from < 0.2 to levels still indicative of photoinhibition ($F_v/F_m = 0.62$) at week 10. Maximal values of this treatment were not reached until week 20. No leaf photodamage was associated with low F_v/F_m. Prior to week 5, F_v/F_m of NS *E. nitens* seedlings was significantly less ($p < 0.001$) than that of other *E. nitens* treatments. From week 10 there were no significant differences between *E. nitens* treatments.

F_v/F_m of both N-Sh *E. globulus* and N-Sh *E. nitens* decreased markedly following planting and were significantly lower 1 ($p < 0.001$) and 4 ($p < 0.05$, $p < 0.001$

respectively) weeks after planting compared to shaded seedlings (Figure 3.3b). In contrast, F_v/F_m of Sh *E. globulus* and Sh *E. nitens* seedlings gradually increased ($p < 0.01$, $p < 0.05$ respectively) from week 1 to week 5. Levels were similar between shaded and non-shaded conspecifics at weeks 5 and 10. At week 16 both shaded treatments appeared to have lower F_v/F_m levels compared to non-shaded treatments, but only Sh *E. globulus* was significantly lower ($p < 0.001$).

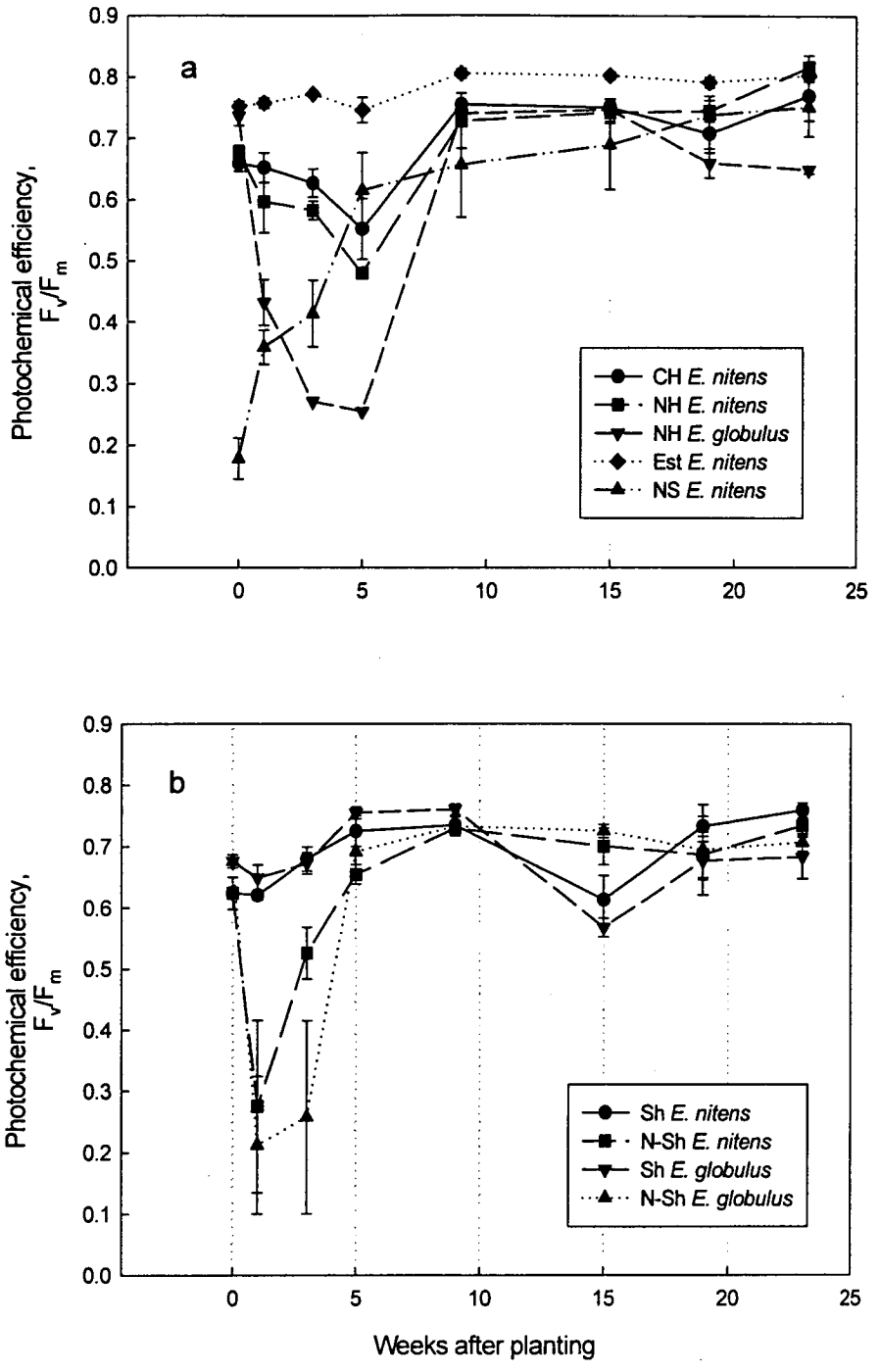


Figure 3.3. Changes in photochemical efficiency (F_v/F_m) of (a) CH, NH and NS *E. nitens* and NH *E. globulus* compared to those of established *E. nitens* saplings (Est. *E. nitens*) and (b) Sh and N-Sh *E. nitens* and *E. globulus* over a period of 23 weeks from planting of the seedlings.

Gas exchange

Changes of A_{max} of CH *E. nitens*, NH *E. nitens* and NH *E. globulus* seedlings were similar to each other throughout the experiment (Figure 3.4a). Linear regression analysis showed that rates of increase of A_{max} with time were similar (ie. no significant differences in slope) but with differing Y intercepts ($p < 0.001$) in order of magnitude CH *E. nitens* (0.0048)> NH *E. nitens* (0.0025)> NH *E. globulus* (0.0007). These relationships differed significantly ($p < 0.001$) from that for established *E. nitens* saplings in which A_{max} remained high and relatively constant (12-17 $\mu mol\ m^{-2}\ s^{-1}$) throughout the experiment. A_{max} of CH *E. nitens* was greater than that of NH *E. nitens* ($p < 0.05$) seedlings at weeks 4 and 5. A_{max} of NH *E. globulus* seedlings decreased after planting and was significantly less ($p < 0.05$) than that of other treatments at weeks 4 and 5. A_{max} of *E. globulus* increased markedly after week 5 and by week 10 was similar to levels in the *E. nitens* seedlings. There were no significant differences in A_{max} between seedling treatments after week 10. A_{max} was similar between seedlings and established *E. nitens* saplings after week 16.

The response of quantum yield (ϕ) for all treatments followed the same trends as A_{max} except for week 4 when ϕ of CH and NH *E. nitens* seedlings were not significantly different (Figure 3.4b). Maximum quantum yields ($\phi = 0.019 - 0.024$) occurred at week 24.

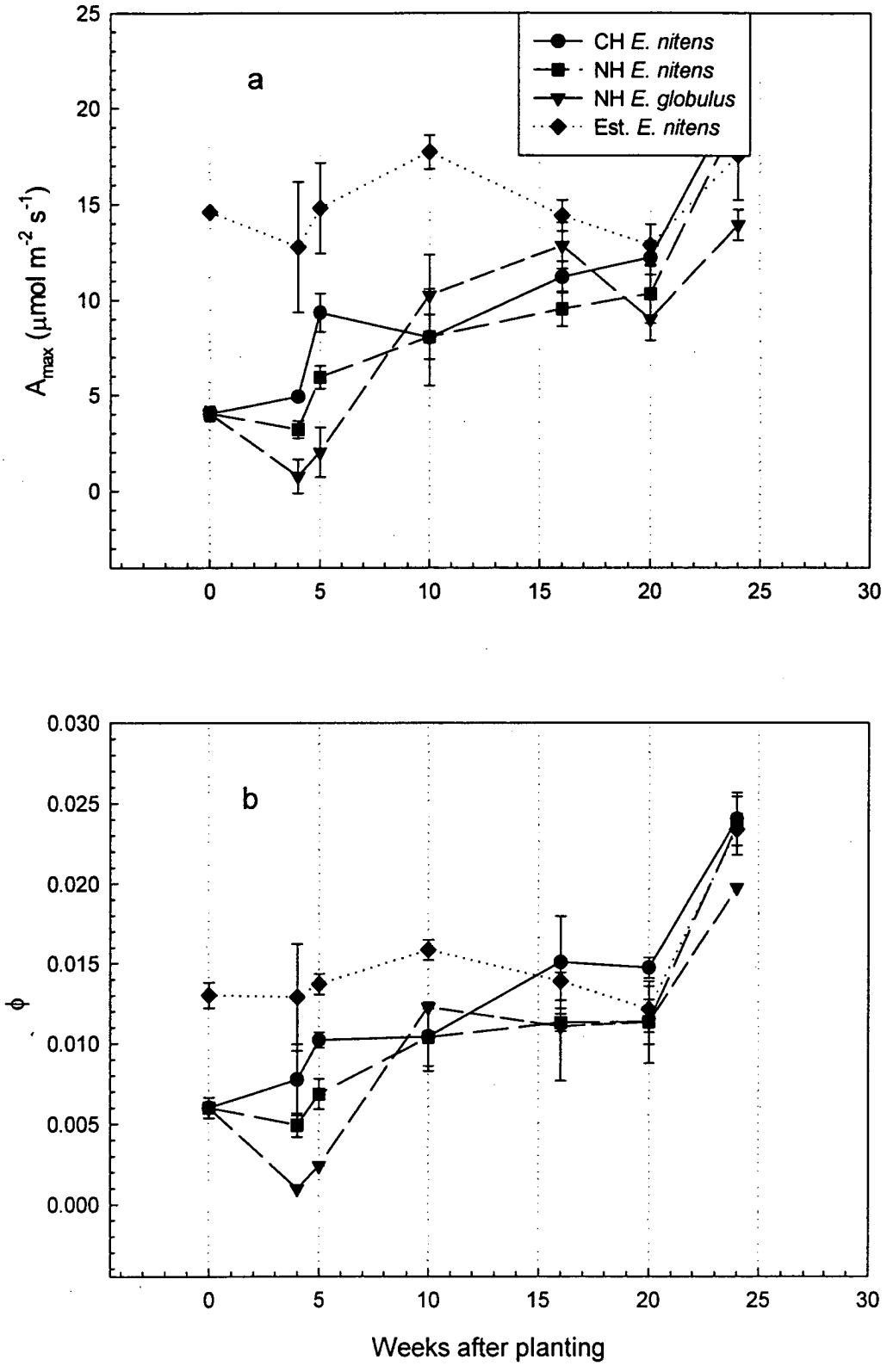


Figure 3.4. Changes in maximum photosynthesis, A_{max} (a) and apparent quantum yield, ϕ (b). Comparisons between treatments are similar to those made in Figure 3.3a but do not include NS *E. nitens*.

Pigments

Total chlorophyll was significantly higher ($p < 0.001$) in established *E. nitens* saplings compared to the seedling treatments at all weeks except week 12 (Figure 3.5a). NS *E. nitens* had significantly less ($p < 0.001$) total chlorophyll at planting and week 5 compared to CH *E. nitens* and NH *E. globulus* seedlings. There was no significant difference between the latter treatments. Total chlorophyll increased between weeks 5 and 12 in all seedling treatments.

Chlorophyll *a*:*b* ratios of seedling treatments (Figure 3.5b) generally decreased between planting and week 5 but then increased and remained at a high level between weeks 9 and 12. There were no significant changes in chlorophyll *a*:*b* of the established *E. nitens* saplings throughout the measurement period. At week 5, NS *E. nitens* had lower chlorophyll *a*:*b* ratios ($p < 0.05$) than other treatments.

The total carotenoid pool of the established *E. nitens* saplings decreased between planting and week 5 but otherwise remained constant throughout the measurement period (Figure 3.5c). At planting, the established *E. nitens* saplings and CH *E. nitens,* NH *E. nitens*, and NS *E. nitens* had significantly different ($p < 0.0001$) carotenoid pool sizes with a rank of Est. = CH > NH > NS *E. nitens*. The pool size of NS *E. nitens* remained significantly lower than those of the other treatments at week 5 ($p < 0.05$). Carotenoid pool sizes had largely converged by week 9.

Leaf anthocyanin levels were significantly higher ($p < 0.01$) in NS *E. nitens* and significantly lower ($p < 0.01$) in NH *E. globulus* seedlings than levels in CH *E. nitens* seedlings and established *E. nitens* saplings at planting (Figure 3.5d). Anthocyanin

levels increased significantly between pre-planting and week 5 in NH *E. globulus* and CH *E. nitens* seedlings: in NH *E. globulus* seedlings the increase was greater than four fold. After week 5, leaf anthocyanin levels decreased and were not significantly different for any treatment.

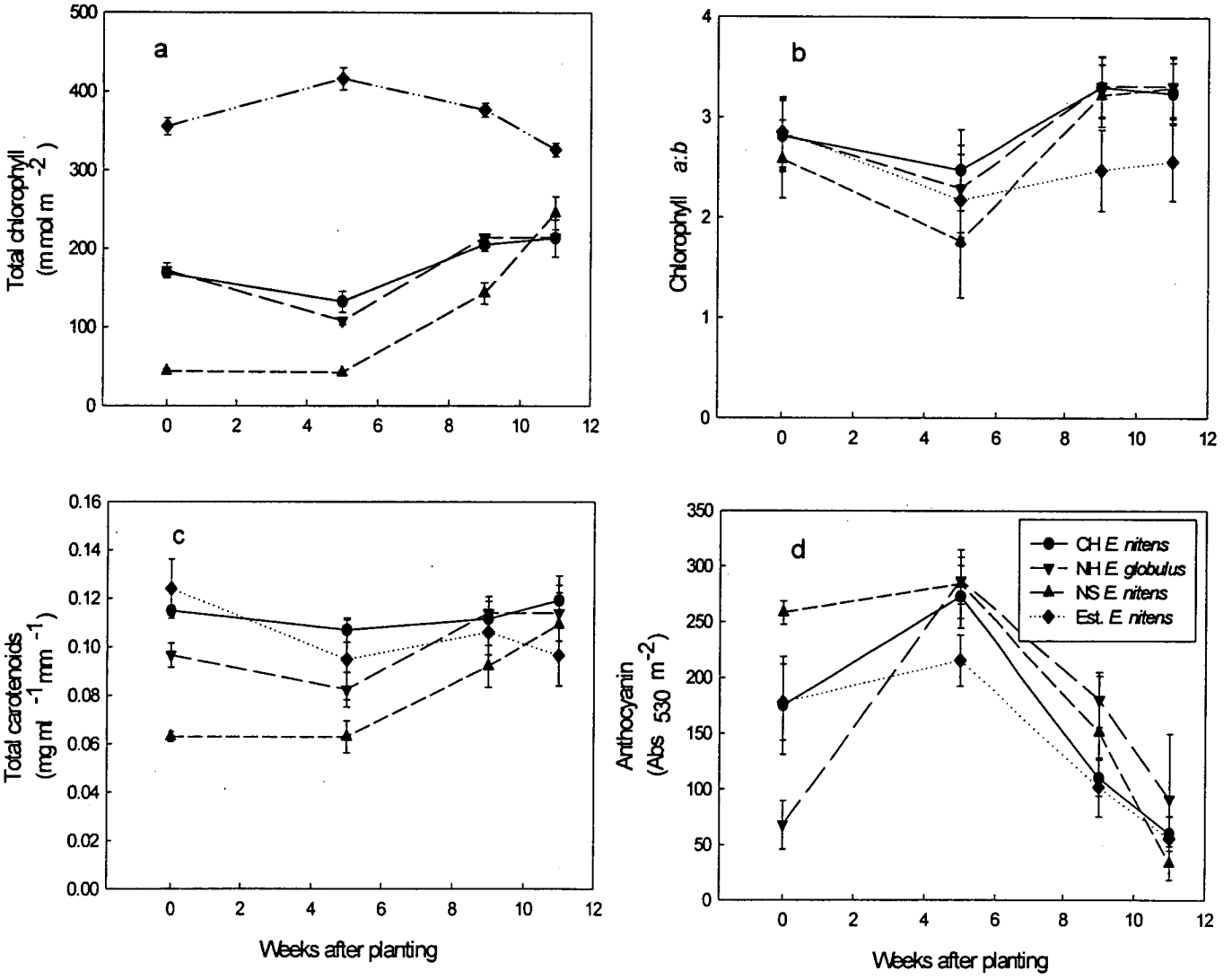


Figure 3.5. Variation during seedling establishment of total chlorophyll (a), chlorophyll *a*:*b* ratio (b), total carotenoids (c), and anthocyanin (d).

Growth Analysis

Height

The heights of NH *E. globulus* and NS *E. nitens* seedlings were significantly ($p < 0.001$) greater than those of CH and NH *E. nitens* at planting (Figure 3.6a). At age 5 weeks, the CH and NH *E. nitens* seedlings had increased in height ($p < 0.001$): the NH *E. globulus* and NS *E. nitens* seedlings showed no increase during this period.

All *E. nitens* seedlings were actively growing by age 9 weeks, although growth of NS *E. nitens* was slower than that of CH and NH *E nitens.* At age 12 weeks and until the end of the experiment there were no significant differences in heights between treatments of *E. nitens* seedlings. A significant increase in height ($p < 0.05$) was not recorded in NH *E. globulus* seedlings until age 16 weeks. Height was significantly less ($p < 0.001$) in NH *E. globulus* than *E. nitens* seedling treatments aged 9 weeks until the end of the experiment. Height of established *E. nitens* saplings increased (increment ~ 200 cm) throughout the experiment (data not shown).

Heights of Sh and N-Sh *E. globulus* seedlings were significantly greater ($p < 0.001$) than those of Sh and N-Sh *E. nitens* seedlings at planting (Figure 3.6b). After planting, Sh and N-Sh *E. nitens* showed similar and positive growth throughout the measurement period. Height growth of Sh *E. globulus* was positive and at a rate similar to that of *E. nitens* treatments until week 16, after which it decreased relative to that of *E. nitens* seedlings. N-Sh *E. globulus* had no positive height growth until age 16 weeks. Height growth of N-Sh *E. globulus* was significantly less ($p < 0.001$) than that of Sh *E. globulus* at age 9 weeks. At age 24 weeks, height of N-Sh *E.*

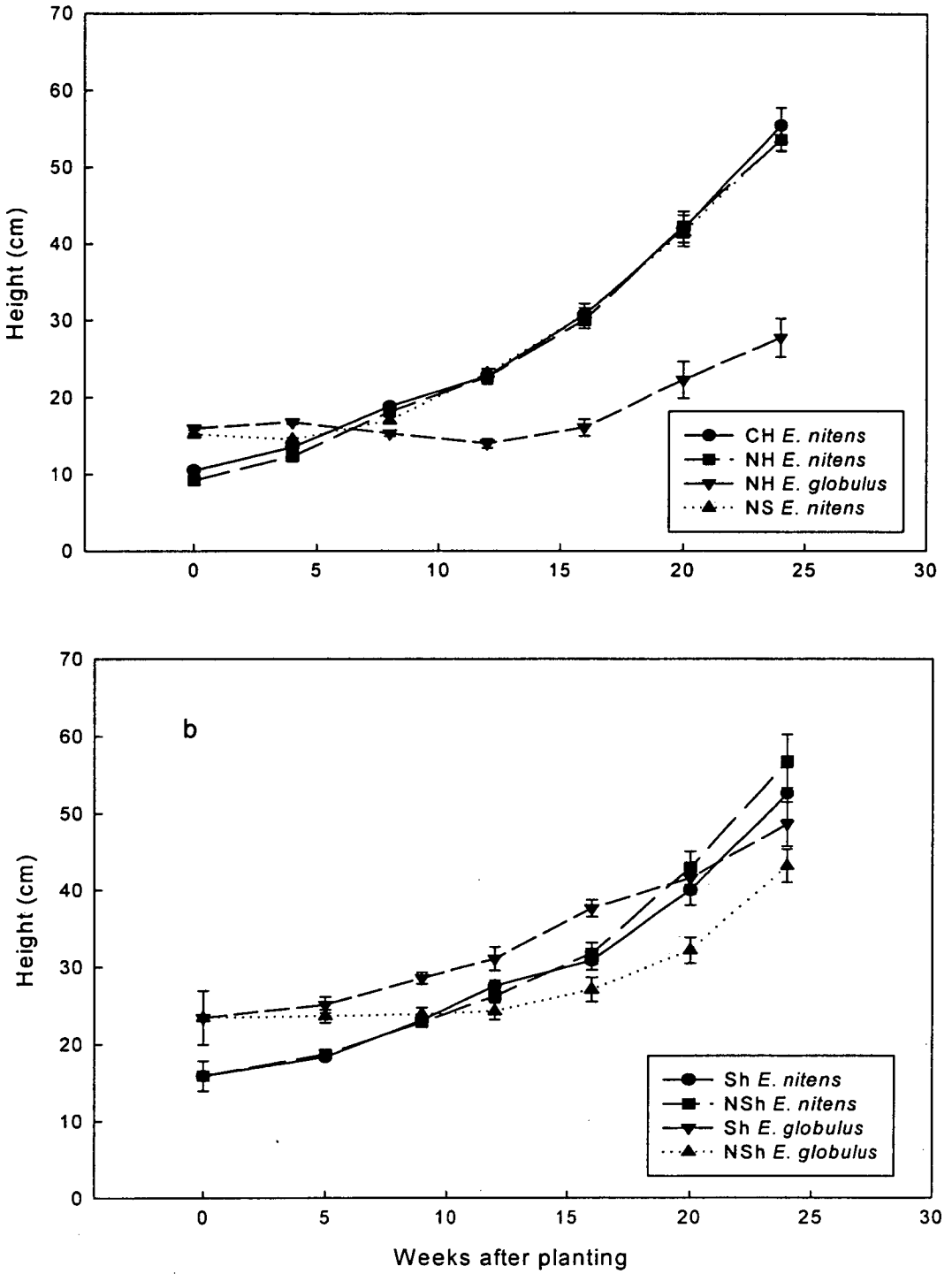


Figure 3.6. Height profile (cm) during seedling establishment. Comparisons between treatments are similar to those made in Figure 3.3a but do not include Est *E. nitens*.

globulus was significantly lower than that of N-Sh or Sh *E. nitens* ($p < 0.002$, $p < 0.02$ respectively) but similar to that of Sh *E. globulus*.

Relative growth rate (RGR)

Relative growth rates increased in all treatments between planting and age 20 weeks and then decreased between age 20 and 47 weeks (Figure 3.7a). RGR of NH *E. globulus* was negative at age 12 weeks and significantly lower at age 9 and 12 weeks ($p < 0.005$, $p < 0.01$ respectively) than that of CH and NH *E. nitens*. There were no significant differences between treatments at age 20 weeks. CH *E. nitens* had significantly higher ($p < 0.001$) RGR at age 47 weeks than in the other treatments.

Net assimilation rate (NAR)

Net assimilation rate was relatively constant and similar for CH and NH *E. nitens* throughout the experiment (Figure 3.7b). However, NAR of NH *E. globulus* was significantly lower ($p < 0.0001$) and higher ($p < 0.001$) than *E. nitens* treatments at weeks 9 and 12, respectively. NAR of all treatments was similar at weeks 20 and 47.

Leaf area ratio (LAR)

After an initial decrease ($p < 0.05$) of LAR in NH *E. globulus*, all treatments increased ($p< 0.05$) LAR from planting until age 20 weeks (Figure 3.7c). From 20 weeks all treatments decreased ($p< 0.001$) to age 47 weeks after planting. At age 12 weeks NH *E. globulus* had significantly lower LAR ($p< 0.005$) compared to other treatments.

Specific leaf area (SLA)

SLA of all treatments generally increased from planting until 20 weeks after planting then decreased by age 47 weeks after planting. At planting, NH *E. nitens* had significantly greater ($p < 0.001$) SLA compared to NH *E. globulus* which in turn had greater ($p < 0.001$) SLA than CH *E. nitens* (Figure 3.7d). At age 9 weeks CH and NH *E. nitens* had similar SLA which was significantly higher than NH *E. globulus* ($p < 0.001$). There were no significant differences in SLA between treatments at age 12 and 20 weeks. At age 47 weeks, SLA of NH *E. globulus* was significantly greater ($p < 0.001$) than in the other treatments. Stratification of SLA with seedling height was observed in seedlings at planting. Leaves developed on low nodes during cool conditions had higher SLA compared with upper nodal leaves which developed during warmer conditions.

Leaf weight ratio (LWR)

There were no significant differences between treatments at planting, and ages 20 and 47 weeks. LWR of NH *E. globulus* was significantly lower ($p < 0.001$) than *E. nitens* treatments at ages 9 and 12 weeks (Figure 3.7e).

Dry weight allocation

The root:shoot ratio of NH *E. globulus* increased significantly ($p < 0.001$) from age 0 to 9 weeks then decreased significantly to age 12 ($p < 0.01$) and 20 weeks ($p < 0.001$) (Figure 3.7f). In contrast, root:shoot ratios of NH *E. nitens* decreased significantly from age 0 to 9 ($p < 0.05$) and from age 12 to 20 weeks ($p < 0.001$). The root:shoot ratios of NH *E. globulus* were higher than those of NH *E. nitens* at age 9 ($p < 0.0001$),

12 ($p < 0.0001$) and 20 ($p < 0.01$) weeks. Differences were not significant at planting or at age 47 weeks.

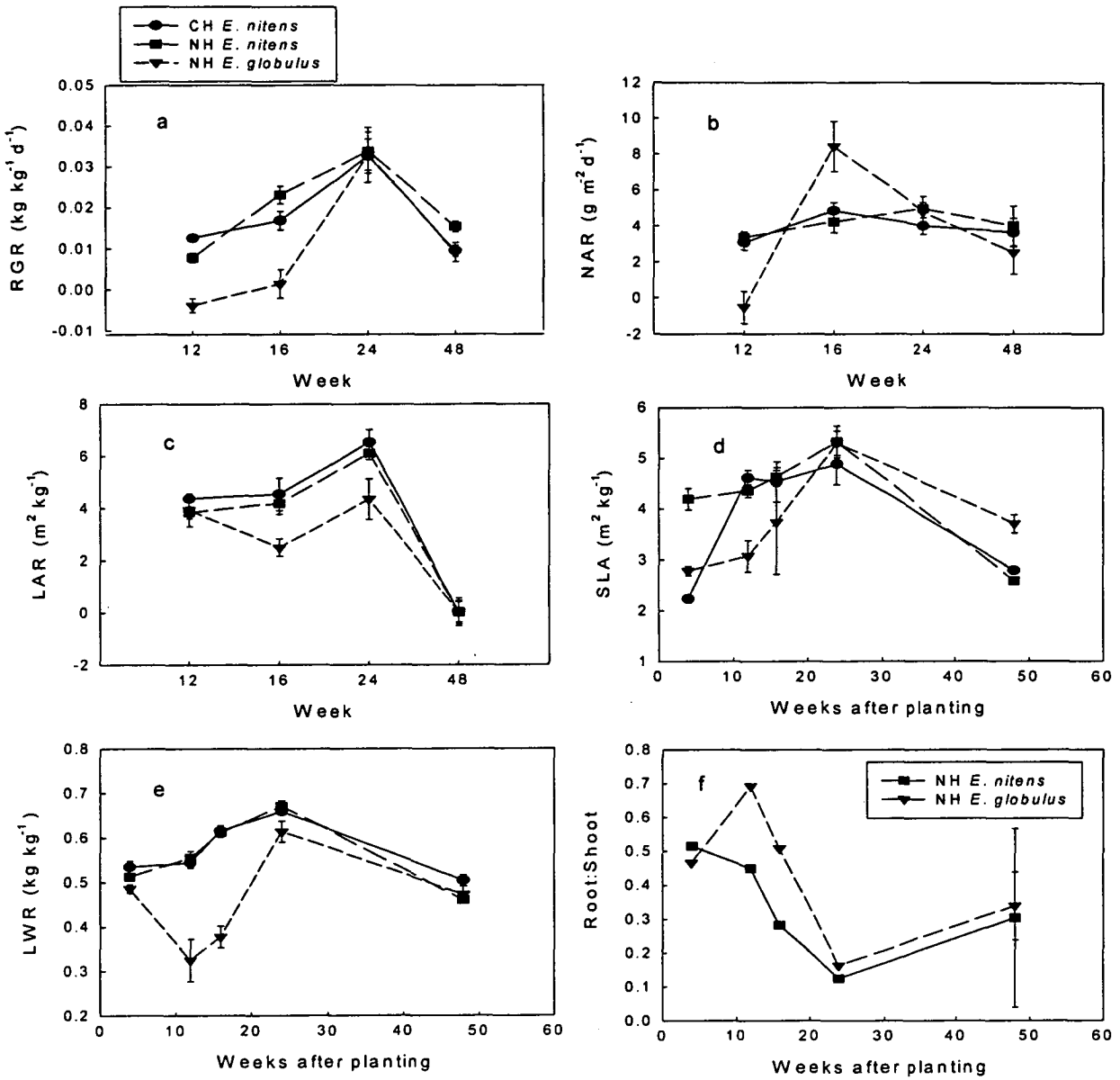


Figure 3.7. Growth analysis variables: (a) relative growth rate (RGR), (b) net assimilation rate (NAR), (c) leaf area ratio (LAR), (d) specific leaf area (SLA), (e) leaf:weight ratio (LWR) and (f) root:shoot ratio during seedling establishment. a-e include the CH and NH *E. nitens* and NH *E. globulus* treatments, f the NH *E. nitens* and *E. globulus* treatments only.

Discussion

Major findings

This work has shown that frost damage and water stress are probably not the prime factors contributing to the survival or performance of *E. globulus* or *E. nitens* seedlings after planting in cold, moist environments. It has also shown that newly transplanted seedlings, regardless of hardening pre-treatments, are inherently susceptible after planting to cold-induced photoinhibition relative to established, acclimated *E. nitens* saplings. Shadecloth treeshelters alleviate this susceptibility. Artificial cold hardening by cold nights alone had little effect on the physiology or growth of *E. nitens* seedlings. *E. globulus* seedlings were (unless shaded) more susceptible to cold-induced photoinhibition than *E. nitens* seedlings. Reductions in height growth after planting were measured in all *E. globulus* (unless shaded) but not *E. nitens* treatments. Shadecloth treeshelters removed the effects of photoinhibition on height growth of *E. globulus*. Differences in levels of carotenoids and anthocyanins at planting are proposed as a major factor contributing to species differences in performance. Nutrient starving of seedlings increased susceptibility to cold-induced photoinhibition immediately after planting but high levels of anthocyanins were associated with minimal leaf tissue damage.

Photoinhibition, photosynthesis, photodamage and pigment chemistry

Established *E. nitens* saplings are acclimated to photoinhibition

No photoinhibition was detected in one-year-old *E. nitens* saplings throughout the experimental period. F_v/F_m of these saplings was close to 0.8 at every measurement, a value typical of non-photoinhibited plants of a wide range of species (Björkman and Demmig 1987). A_{max} was also similar to levels reported previously for non-

photoinhibited *E. nitens* (Davidson *et al.* 1995; Battaglia *et al.* 1996). Although established *E. nitens* saplings were approximately three- to six-fold taller than seedlings (Table 3.1), air temperature differences between 0.15, 0.30 and 0.45 m above ground level were negligible (see Appendix 3). These results indicate that the low temperatures experienced during the trial period were not stressful for acclimated individuals of *E. nitens*.

Is damage caused by frost or photoinhibition?

Artificial cold hardening induces a suite of biochemical and physiological changes in plants (Guy 1990; Wanner and Junttila 1999) that act to decrease the low temperature limit at which leaf damage occurs (Guy *et al.* 1987; Gilmour *et al.* 1988). However, the significant difference in frost tolerance at planting between CH *E. nitens* and NH *E. nitens* seedlings was not linked to any observed differences in rates of physiological processes measured in the field. This indicates that frost damage did not occur in *E. nitens* seedlings. In other studies of seedling establishment (eg. Ball *et al.* 1991; Örlander 1993; Holly *et al.* 1994; Germino and Smith 1999), it has been demonstrated that the combination of high irradiances with low temperatures causing cold-induced photoinhibition, and not simply low temperature *per se*, can affect tree seedling performance and mortality in the field. The observation that there was no effect of nutrient starvation on frost tolerance of NS *E. nitens* seedlings was consistent with other studies (Hellergren 1981, Toivonen *et al.* 1991, Hawkins *et al.* 1994).

The role of hardening – CH and NH *E. nitens*

In a controlled-environment study, *E. nitens* seedlings grown at a nocturnal temperature of 3 °C were less photoinhibited at temperatures below 5 °C than

seedlings grown at a nocturnal temperature of 8 °C (Warren *et al.* 1998). However, cold-hardening had little effect in alleviating cold-induced photoinhibition measured in this experiment. This may be because the CH *E. nitens* was cold hardened during winter and the pre-conditioning temperatures were not sufficiently different from those used for the NH *E. nitens* to affect significantly the physiological attributes which alleviate photoinhibition. Levels of chlorophylls, carotenoids and anthocyanins did not differ between these treatments. In effect, the NH *E. nitens* was sufficiently hardened to tolerate the conditions of high light and low temperature experienced during the experiment.

Low chlorophyll levels in seedlings

Immature leaves have been found to have lower chlorophyll levels, lower rates of photosynthesis and higher levels of photoinhibition compared to mature foliage (Krause *et al.* 1995; Dodd *et al.* 1998). The possible benefits of acclimation to photoinhibition, by artificial cold-hardening of the CH *E. nitens* seedlings, may have been lost due to the high inherent susceptibility to photoinhibition of the young seedlings used in the experiment, regardless of treatment.

NH *E. globulus* – ecological links and species differences

A marked photoinhibitory response immediately after planting was apparent for NH *E. globulus* seedlings and was associated with leaf abscission and seedling mortality. Responses to sudden exposure to cold-induced photoinhibitory conditions and capacities for subsequent acclimation to such conditions vary according to species (Ferrar and Osmond 1986; Logan *et al.* 1998; Mohammed and Parker 1999). These differences may depend in part on the capacity of the xanthophyll cycle for dissipating

energy (Demmig-Adams and Adams 1996). This hypothesis is supported by the observation that levels of total carotenoids in *E. nitens* saplings and CH *E. nitens* were greater than in NH *E. globulus*. Thus *E. globulus* may have a lower inherent capacity for the dissipation of excess energy than *E. nitens*. Similarly, species differences in maximal photochemical efficiency (F_v/F_m) have been reported for conifers (Gnojek 1992; Leiffers *et al.* 1993; Zhang *et al.* 1997; Mohammed and Parker 1999). The findings here suggest that cold-induced photoinhibition may be a factor determining the range of environments where *E. globulus* can successfully be planted, in addition to its low frost tolerance (Tibbits 1986). Sensitivity to cold-induced photoinhibition has been suggested to partially explain the natural distribution and habitat niches of *E. pauciflora* (Ball *et al.* 1991; Warren *et al.* 1998), *E. nitens* (Warren *et al.* 1998) and *Ilex aquifolium* (Groom *et al.* 1991).

Alleviating photoinhibition with shade

The hypothesis that cold-induced photoinhibition is a factor affecting the planting distribution of *E. globulus* and that greater levels of carotenoids in *E. nitens* relative to *E. globulus* confer greater tolerance to cold-induced photoinhibition through excess light energy dissipation is supported by the results of the shadecloth tree shelter treatments. F_v/F_m of Sh *E. globulus* and Sh *E. nitens* seedlings increased after planting while F_v/F_m of all exposed treatments, except NS *E. nitens*, decreased. Shadecloth tree shelters had no effect on seedling temperature. Thus high light levels caused severe cold-induced photoinhibition in N-Sh *E. globulus* and 50% shadecloth was more effective in preventing that photoinhibition in *E. globulus* than excess energy dissipation by carotenoids was in preventing photoinhibition in CH and NH *E. nitens*. Reduced incident light levels under an established tree canopy (Ball *et al.*

1991; Ball 1994; Germino and Smith 1999), changes in leaf orientation (Lundmark and Hällgren 1987) as well as shadecloth tree shelters (Holly *et al.* 1994) have been demonstrated to decrease the severity of photoinhibition at temperatures associated normally with severe photoinhibition. The decrease in F_v/F_m in N-Sh *E. nitens* following planting was more severe than that observed in CH and NH *E. nitens* due to the differing growing conditions of the stock used (Table 3.1). Nevertheless, leaf damage in N-Sh *E. nitens* compared to N-Sh *E. globulus* was minimal. F_v/F_m decreased following shadecloth removal. This decrease was small relative to that induced by frost and indicative of the warmer, early summer, conditions prevalent at week 14. However, anthocyanin levels still rose. This demonstrates the rapidity of anthocyanin production in eucalypt seedlings after exposure to high light and low temperature conditions.

NS *E. nitens* – a special case

Sustained xanthophyll dependent energy dissipation (ie. low pre-dawn F_v/F_m) following nights of freezing temperatures has been linked to a reduction of photodamage during the following morning under conditions of high light and low temperature (Adams *et al.* 1994). This response was induced in the NS *E. nitens* by nutrient starvation which, by decreasing chlorophyll levels and photosynthetic capacity, increases the excess of excitation energy (Jacob 1995) and decreases the ability of the plant to re-synthesise damaged proteins in photosystem II (Balachandren and Osmond 1994; Godde and Hefer 1994).

Chlorophyll and carotenoid levels are related to photoinhibition

The decrease in total and chlorophyll *a*:*b* levels between planting and 5 weeks after planting in the CH *E. nitens* and NH *E. globulus* seedlings, which is indicative of photo-oxidative stress (MacWilliam and Naylor 1967; Haldimann 1998, 1999), was consistent with the observed decreases of F_v/F_m in these treatments. In NS *E. nitens* seedlings there was no decrease in total chlorophyll or F_v/F_m levels which accords with the lack of visible damage in this treatment relative to other seedling treatments.

Plants resistant to cold-induced photoinhibition commonly have higher levels of carotenoids (Demmig-Adams and Adams 1996; Haldimann 1999). Such a relationship was observed in this experiment. The order of magnitude of carotenoid pools at planting was established *E. nitens* saplings > CH *E. nitens* > NH *E. globulus* > NS *E. nitens* seedlings and this corresponded to the relative degree of photoinhibition as measured by F_v/F_m. It is notable that NH *E. globulus* had lower levels of carotenoids at planting and week 5 compared to CH *E. nitens*. Thus carotenoid and chlorophyll levels were related to the degree of photoinhibition experienced. Increased levels of carotenoids have often been reported as a consequence of prolonged exposure to photoinhibition (Adams *et al.* 1994; García-Plazaola *et al.* 1999a). However, nutrient starvation did not increase levels of carotenoids in NS *E. nitens* seedlings which remained in the same proportion to chlorophylls as in other treatments.

A possible role for anthocyanins in decreasing cold-induced photoinhibition

The presence of anthocyanins in young eucalypt leaves has been reported previously (Sharma and Crowden 1974) but was not related to specific environmental conditions. Increased synthesis of anthocyanin has been reported in *Pinus sylvestris* L. seedlings following early frosts (Nozzolillo *et al.* 1989), similar to the responses of CH *E. nitens* and NH *E. globulus* observed in this trial. Two patterns of anthocyanin levels were apparent after planting. In CH *E. nitens* and NH *E. globulus* anthocyanin levels increased. This increase was associated with decreased total chlorophyll levels and increased photoinhibition, and with moderate and severe visible leaf damage, respectively. In contrast, anthocyanin levels of established *E. nitens* saplings and NS *E. nitens* seedlings remained constant. This was associated with unchanged chlorophyll levels in both treatments, no change in photoinhibition of established saplings and a marked decrease in photoinhibition of NS *E. nitens*. No visible leaf tissue damage was observed in either treatment.

This combination of results indicates strongly that anthocyanin synthesis is induced by environmental conditions that result in photoinhibition. Established *E. nitens* saplings and NS *E. nitens* seedlings were acclimated to the conditions prevailing between planting and 5 weeks after planting. After week 5 anthocyanin was gradually desynthesised in parallel with the gradual increase in temperatures and photosynthetic rates. This phenomenon has been observed in *P. sylvestris* seedlings after removal to a warm greenhouse (Nozzolillo *et al.* 1989), although in this case it was not associated with photoinhibition.

Accumulation of anthocyanins in *P. sylvestris* seedlings in response to nutrient starvation at mild temperatures has also been reported previously (Nozzolillo *et al.* 1989; Toivenon *et al.* 1991). These, as yet, unexplained observations and the results from this experiment are however consistent with current ecophysiological evidence that indicates a role for anthocyanins in light attenuation (Krol *et al.* 1992; Barker *et al.* 1997; Dodd *et al.* 1998), thereby decreasing cold-induced photoinhibition.

Growth analysis

Height

Height of seedling treatments differed before planting which may have affected subsequent physiological and growth measurements. However, given the lack of cold air stratification on mornings following frosts between 0.15, 0.30 and 0.45 m above ground level (see Appendix 3), it is unlikely that stratification occurred between 0 and 0.15 m above ground level.

Severe photoinhibition and photodamage soon after planting was associated with no positive height growth of NH *E. globulus* until age 16 weeks. Similarly, impaired growth was observed in *E. polyanthemos* and *Pinus ponderosa* Laws under conditions of severe depression of photosystem II efficiency (Adams *et al.* 1994; Holly *et al.* 1994). In contrast, moderate photoinhibition had no effect on CH and NH *E. nitens* and positive height growth was observed 4 weeks after planting. No growth occurred in NS *E. nitens* until F_v/F_m had recovered from very low levels. However by age 8 weeks photoinhibition and height growth were similar to those of other *E. nitens* treatments. This is consistent with growth of photoinhibited *Pinus banksiana* Lamb.

upon transfer to non-photoinhibitory conditions in a greenhouse (Nozzolillo *et al.* 1989).

Shading increased height growth of *E. globulus* but not *E. nitens* confirming that cold-induced photoinhibition can reduce growth of *E. globulus*. Height growth of *E. globulus* was impaired also following shadecloth removal. Thus sudden changes in incident light can affect the growth of plants with limited capacity for excess energy dissipation. Similar effects on growth have been documented following sudden increases in incident light in *Fagus sylvatica* L. (Tognetti *et al.* 1998) and *Tsuga canadensis* (L.) Carr. (Mohammed and Parker 1999).

Relative growth rate (RGR)

RGR is the product of the net assimilation rate per unit leaf area (NAR) and the leaf area ratio (LAR). LAR changed in response to levels of incident light (Poorter 1999), and to changes in morphology eg. phyllodes within species (Atkin *et al.* 1998) and differed between shade tolerant and intolerant species (Niinemets 1998). NAR is altered by the effects of stress on photosynthesis eg. cold temperature (Dunn *et al.* 1987); viral pathogen infection (Navas *et al.* 1998); herbicide (Kremer *et al.* 1999); salinity (Ruiz *et al.* 1997) and weed competition for nutrients and water (Brand 1991; Britt *et al.* 1991). Changes in RGR with time and between species between planting and age 24 weeks were caused initially by differences in NAR (week 12) and later by differences in LAR (weeks 16). The lower RGR and NAR of NH *E. globulus* at week 12 were due to cold-induced photoinhibition and at 16 weeks after planting resulted from leaf abscission subsequent to frost events during the first 3 weeks after planting. RGR of stressed relative to non-stressed treatments can be similar due to

compensatory increases in NAR when LAR has decreased (Brand 1991; Kremer *et al.* 1999). However this was not observed in NH *E. globulus*.

Leaf abscission reduced LWR and LAR of NH *E. globulus* between planting and week 24. Low SLA of NH *E. globulus* at week 12 was because upper nodal, higher SLA leaves (developed during mild conditions) abscised soon after planting. Thus lower retained leaves of low SLA which developed during cool temperatures were sampled at that time. Low SLA is associated with growth under cool conditions (Robson and Jewiss 1968; Hillard and West 1970; Woodward 1979).

LWR of NH *E. globulus* 12, 16 and 24 weeks after planting was reduced also by increased root:shoot ratio. Increased diversion of dry weight to below ground organs has been found to result from nutrient stress (eg. Cromer and Jarvis 1990) and this may have played a role in leaf abscission. Reduced LARs have similarly been reported to result in differences in RGR of different species of establishing rain-forest tree seedlings under low light (Poorter 1999). However, reduced NAR has been found to affect RGR of established seedlings in high light (Yadav *et al.* 1986). This could conceivably occur in NH *E. globulus* seedlings given less extreme cold-induced photoinhibition over a longer period.

Conclusion

It has been shown that cold-induced photoinhibition may restrict the establishment of *E. globulus* on a site considered marginal for planting this species but that artificial shading can mitigate this effect. Comparisons between CH and NH *E. nitens* treatments indicated no benefit of artificial cold hardening in terms of subsequent

growth after planting. A similar comparison between Sh and N-Sh *E. nitens* indicated no benefit of shading *E. nitens* seedlings at this site. Hardening seedlings by withholding nutrients prevented further decrease of F_v/F_m during establishment. This may have been due to sustained overnight xanthophyll engagement and/or possible attenuation of incident light by anthocyanins. Increased anthocyanin synthesis occurred as a result of cold-induced photoinhibition after planting and shadecloth tree shelter removal in the non nutrient-starved seedlings, the degree of photoinhibition correlating to the relative increases in anthocyanin levels. In contrast, established *E. nitens* saplings experienced no cold-induced photoinhibition and no increase in anthocyanin levels. Most importantly, pre-conditioning for photoinhibition through nutrient starvation had no long term effect on growth performance on spring-planted seedlings, suggesting this as a practical and useful risk management tool in seedling production for eucalypt plantation forestry in cold environments.

Given the well established relationship between photosynthesis and foliar N and P and: a) the large differences in tolerance to cold-induced photoinhibition between *E. globulus* and *E. nitens*; b) reports of higher foliar N in *E. nitens* induced by artificial cold-hardening and; c) the rapid recovery of photosynthetic rate of NS *E. nitens*, it is of interest to investigate seedling nutrition physiology during establishment. This is the subject of investigation in the following chapter.

Chapter 4. Partitioning of nitrogen and phosphorus chemical fractions in *E. nitens* and *E. globulus* seedlings after an early spring planting

Introduction

Foliar N and P and photosynthesis

Correlations between total foliar N and P and photosynthesis have been reported in various eucalypt species (Field and Mooney 1986; Mulligan 1989; Kirschbaum and Tompkins 1990; Sheriff and Nambiar 1991). However, this broad relationship can only be interpreted in a limited way as carbon assimilation depends on N partitioning within the leaf (Evans 1989). N partitioning is affected by many environmental factors (Field *et al.* 1983). Thus, a more sensitive method of assessing foliar N and/or P sufficiency or deficiency for interpreting their effects on photosynthesis and growth is to separate physiologically active forms from those which are structural.

Chemical fractionation

A decrease in inorganic P, via phosphorus deprivation in solution culture, was shown not to limit photosynthesis in *Eucalyptus grandis* Hill ex Maiden, *E. pilularis* Smith and *E. gummifera* (Sol. ex Gaertner) Hochr. seedlings (Mulligan 1989). Chemical fractions of foliar N and P were also found to provide superior detail compared to total N and P analysis in investigations of nutrient cycling in plantations of *Pinus taeda* L. and *Pinus elliottii* Engelm. var. elliottii and *Eucalyptus globulus* (Polglase *et al.* 1992a; 1992b; Hooda and Weston 1999).

Foliar N and P and cold hardening

Reduced sensitivity to photoinhibition following cold hardening of *Seceale cereale* L. cv Musketeer has been reported through inorganic phosphate feeding (Hurry *et al.* 1993). However a similar investigation reported no such effect on *Eucalyptus pauciflora* and *E. nitens* (Warren 1996). Increased organic N associated with photosynthetic enzymes has been linked with increased photosynthetic capacity during growth under low temperatures in cold-tolerant *Triticum aestivum* (Hurry and Huner 1991). Both increased foliar N and photosynthetic capacity were reported for cold hardened *E. pauciflora* and *E. nitens* (Warren 1996; Hovenden and Warren 1998; Warren *et al.* 1998).

Nursery practice

Boom fertigation (application of soluble fertilisers via boom irrigators) in nurseries allows precise control of the application of soluble fertilisers to seedlings. Nutrient loading, the application of nutrients at levels higher than that required for growth just before leaving the nursery, allows luxury consumption of nutrients by seedlings. This has been shown to increase relative growth rate of *Pinus resinosa, Picea mariana* and *Picea glauca* seedlings on both herbicide-treated and untreated planting sites compared to non-nutrient-loaded seedlings (Miller and Timmer 1994; 1997; Malik and Timmer 1995; McAllister and Timmer 1998; Timmer 1999). In contrast, pre-conditioning seedlings in the nursery by withholding nutrients has been used for the production of seedlings of *Eucalyptus regnans*, *Eucalyptus fastigata* and *E. nitens* suitable for planting on frosty sites (Forest Research Institute 1987).

Hypotheses tested

In the experiment described in this chapter, chemical fractions of fertilised and nutrient-starved *E. nitens* and fertilised *E. globulus* were investigated and their effects on photosynthesis and growth assessed. Three hypotheses are proposed and tested;

- that CH *E. nitens* will have greater foliar organic N and photosynthetic levels compared to NH *E. nitens*:
- that the higher nutrient status of CH and NH *E. nitens* will support higher levels of photosynthesis and growth relative to nutrient-starved NS *E. nitens* and;
- that the nutrient status of NH *E. globulus* will not support higher levels of photosynthesis and growth compared to NH *E. nitens* treatments due to its relative intolerance to cold-induced photoinhibition (incorporating results from Chapter 3).

Materials and methods

General

Site description, plant material, experimental design, gas exchange and height and growth analysis were as described in Chapter 3.

Nutrient analysis

The procedures followed were as described in Chapter 2. Material for analysis of CH and NH *E. nitens* and NH *E. globulus* was sampled on 14 August, 14 September, 18 November, 10 December 1997 and 4 February 1998 (0, 1, 9, 13 and 20 weeks after planting respectively). NS *E. nitens* was sampled only on 14 September and 18 November 1997.

Statistical analysis

Differences in reported variables were analysed as detailed in Chapter 2.

Results

Nutrient analysis

Total foliar nitrogen

NH *E. globulus* foliage had 2.9 % N at planting, significantly higher ($p < 0.001$) than that of NH *E. nitens* (2.0 %) which was higher ($p < 0.05$) than that of CH *E. nitens* (1.6 %). One week after planting total N had decreased significantly in all treatments. CH *E. nitens* had significantly higher ($p< 0.05$) N levels (1.3 %) than NH *E. globulus* (1.1 %) and NH *E. nitens* (1.1 %) (Figure 4.1a). Total N of all treatments increased significantly ($p < 0.01$) from week 1 until week 20. From week 7 NH *E. globulus* had higher ($p < 0.05$) N compared to CH and NH *E. nitens*. NS *E. nitens* had lower levels of N compared to other treatments ($p < 0.001$). It was 0.4 % N at week 1 and 1.1 % at week 7.

Nucleic acid N

At planting, nucleic acid N in CH *E. nitens* (0.07 %) was significantly lower ($p < 0.005$) than in NH *E. globulus* (0.21%) and NH *E. nitens* (0.17 %). (Figure 4.1b). In all three treatments it was 0.10 ± 0.03 % at week 1. Levels remained relatively constant through weeks 9 and 13 and there were no significant differences between treatments. Levels then increased ($p < 0.005$) to 0.18 ± 0.05 % at week 20. At week 1 NS *E. nitens* had significantly lower (0.02 %) ($p < 0.01$) levels of nucleic acid N than in other treatments but this had increased to similar levels by week 9.

Soluble (nitrate, ammonia and amino acid) N

At planting, soluble N in CH *E. nitens* (0.03%) (Figure 4.1c) was significantly lower ($p < 0.0050$) than in NH *E. globulus* (0.11%) and NH *E. nitens* (0.07%). At week 1 all three treatments had similar levels of soluble N (0.05 % ± 0.005). Levels increased to 0.07 % ± 0.003 at week 9 ($p > 0.05$) and to 0.11 % ± 0.004 at week 13 ($p < 0.05$). Levels of soluble N in NS *E. nitens* were significantly lower ($p < 0.01$) than in other treatments at week 1 but were similar by week 9.

Protein N

Protein N consistently accounted for 85-95% of total N. Accordingly it followed exactly the same pattern of changes with time as that of total N (Figure 4.1d).

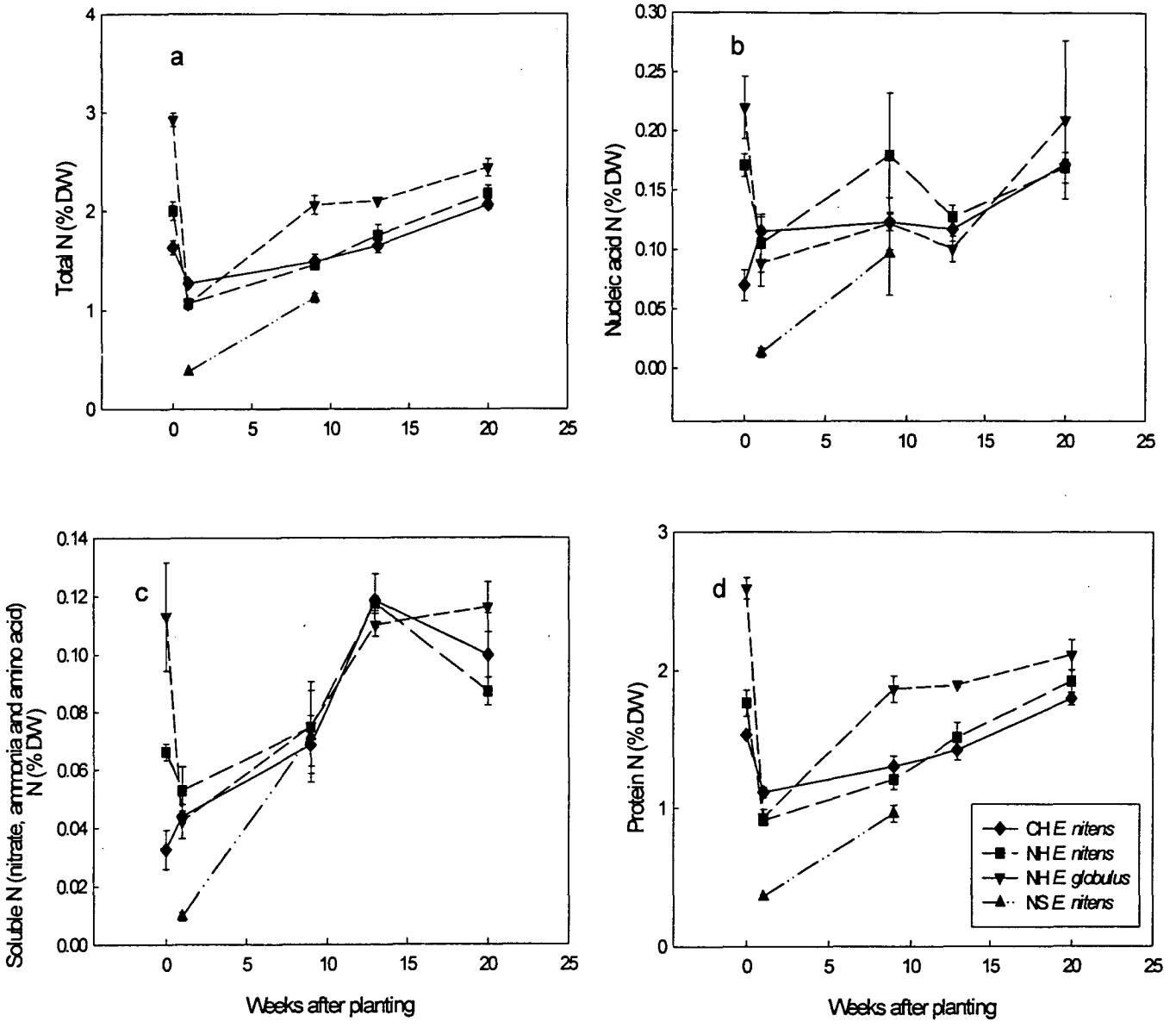


Figure 4.1. Changes in (a) total N, (b) nucleic acid N, (c) soluble N and (d) protein N over a period of 20 weeks from planting of the seedlings.

Total phosphorus

At planting, total P in NH *E. globulus* (0.19 %) was significantly higher ($p < 0.001$) than that of either CH or NH *E. nitens* which were similar (0.11 % ± 0.025) (Figure 4.2a). Levels had increased ($p < 0.001$) in CH and NH *E. nitens* at week 1 to 0.17 and 0.15%. This level was significantly higher ($p < 0.05$) for CH *E. nitens*. Total P of NH *E. globulus* decreased ($p < 0.05$) between planting and week 1 and was significantly lower ($p < 0.05$) than in *E. nitens* seedlings. Total P generally decreased between week 1 and week 20 in the NH *E. globulus* and CH and NH *E. nitens* treatments NS *E. nitens* had significantly lower P at week 1 (0.05%) but then increased ($p < 0.001$) and was similar (0.115 %) to that of NH *E. globulus* by week 9.

Nucleic acid P

At planting nucleic acid P in NH *E. globulus* (0.046 %) was significantly higher ($p< 0.005$) than in NH *E. nitens* (0.027 %) and CH *E. nitens* (0.020 %) (Figure 4.2b). In CH and NH *E. nitens* and NH *E. globulus*, levels of nucleic acid P were 0.03 % ± 0.003 at week1. These levels remained relatively constant for NH *E. globulus* but in CH and NH *E. nitens* decreased to week 13 ($p < 0.005$). At week 13, nucleic acid P in these *E. nitens* treatments was significantly lower ($p < 0.05$) compared to NH *E. globulus*. NS *E. nitens* had significantly lower ($p < 0.001$) nucleic acid P than other treatments at week 1. Its level increased ($p < 0.05$) and was similar to other treatments at week 9.

Sugar phosphate

Differences in sugar phosphate (organic P) at planting were similar to those of nucleic acid P viz. NH *E. globulus* (0.035%) > NH *E. nitens* (0.019%) > CH *E. nitens*

(0.011%) ($p < 0.01$, $p < 0.08$ respectively) (Figure 4.2c). At week 1, organic P had increased ($p < 0.001$) to 0.042% in CH and NH *E. nitens*, and decreased to 0.025 % ($p < 0.05$) in NH *E. globulus* resulting in significantly higher ($p < 0.0005$) organic P in the CH and NH *E. nitens* compared to NH *E. globulus*. Organic P decreased ($p < 0.001$ and $p < 0.06$ for *E. nitens* and NH *E. globulus* respectively) to around 0.015% at week 9. While levels in NH *E. globulus* remained relatively constant, in CH and NH *E. nitens* it decreased further ($p < 0.001$) to 0.01% at week 13 (similar to nucleic acid levels). Organic P was significantly lower ($p < 0.001$) in NS *E. nitens* compared to NH *E. globulus* at week 13 but similar by week 20.

Inorganic P (P_i)

P_i was similar between treatments at planting (Figure 4.2d). Levels in CH and NH *E. nitens* increased significantly ($p < 0.001$) at week 1 while that of NH *E. globulus* had decreased ($p < 0.001$) and was significantly lower ($p < 0.001$) than that of CH and NH *E. nitens* treatments. CH and NH *E. nitens* decreased ($p < 0.001$) to week 13 as did NH *E. globulus* ($p < 0.001$) but in *E. nitens* seedlings, levels were higher than in NH *E. globulus* at weeks 9 and 13 ($p < 0.01$ and $p < 0.06$ respectively). From week 13-20 levels remained constant in all three treatments. At week 1, P_i in NS *E. nitens* was significantly lower ($p < 0.001$) than in other *E. nitens* seedlings but increased ($p < 0.01$) to be similar at week 9.

Insoluble P complexes

Insoluble P complexes were relatively constant throughout the trial (Figure 4.2e), though that for NH *E. globulus* was consistently higher ($p < 0.05$) compared to those for CH and NH *E. nitens* treatments. NS *E. nitens* was similar to other *E. nitens*

treatments at week 1 but increased to be higher ($p < 0.01$) than these treatments at week 9.

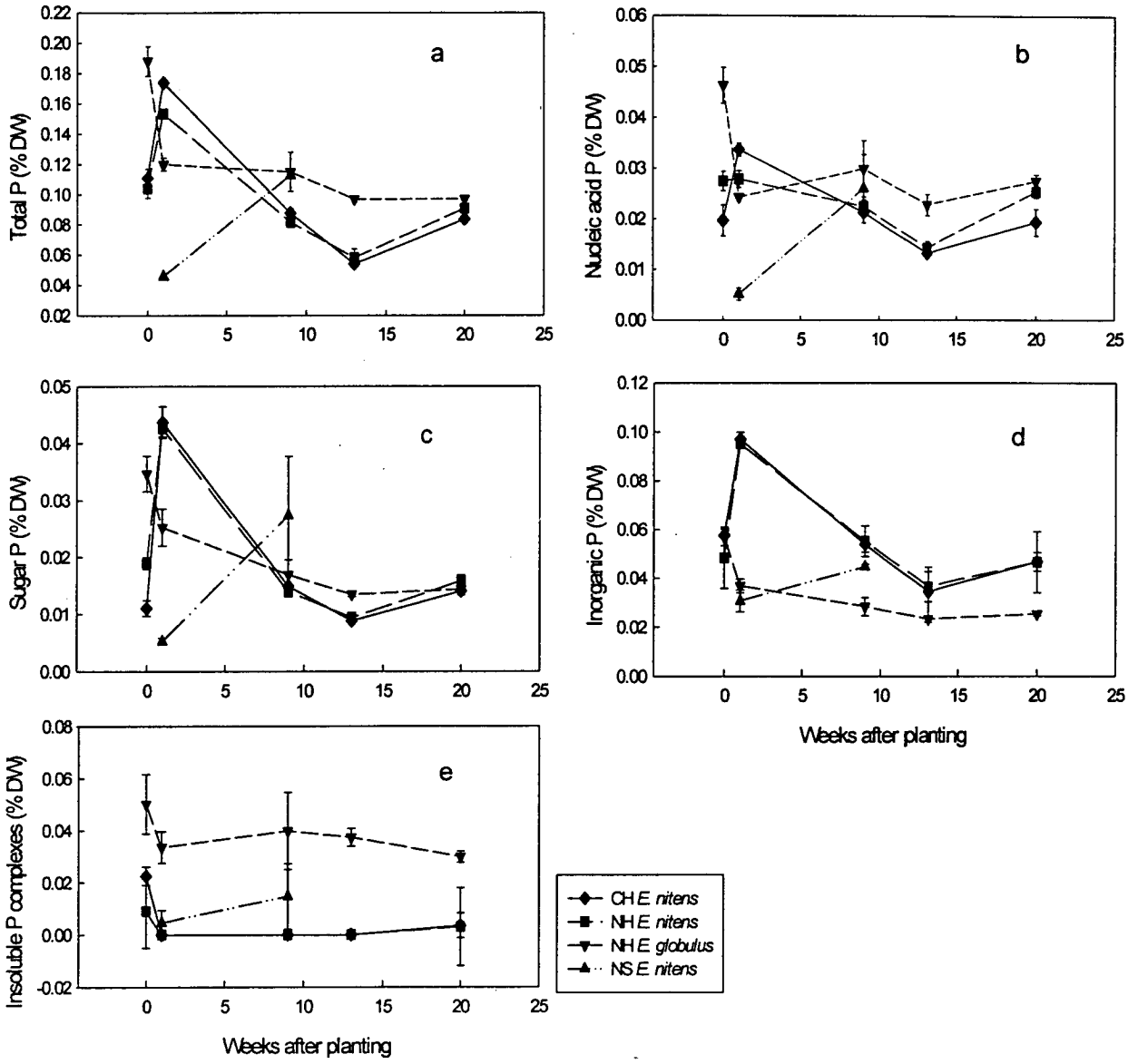


Figure 4.2. Changes in (a) total P, (b) nucleic acid P, (c) sugar P, (d) inorganic P and (e) insoluble P complexes over a period of 20 weeks from planting of the seedlings.

Gas exchange, height and growth analysis

Results of gas exchange, height and growth analyses were presented and described in Chapter 3.

Discussion

Major findings

This work has demonstrated inherent species differences in the nutritional physiology of *E. nitens* and *E. globulus* seedlings, both in the nursery and during establishment. In the nursery, and with added nutrient, *E. globulus* seedlings absorbed greater levels of N and had more vigorous growth relative to *E. nitens* seedlings of the same age. Soon after planting, large differences in soluble and inorganic P fractions were evident which may have been associated with high levels of cold-induced photoinhibition and photodamage in *E. globulus* relative to *E. nitens*. Differences in foliar N between CH, NH *E. nitens* and NH *E. globulus* before planting dissipated within four weeks after planting. These changes were associated with increased root growth and adjustment of shoot:root ratio from nursery to field values. After this initial decrease of N, the levels increased gradually. This was paralleled by an increase in soluble N possibly indicating uptake of soil N. This was most pronounced in NS *E. nitens* which showed the greatest increase in total N.

Allocation of foliar nutrients differs between species

The allocation of foliar nutrients in NH *E. globulus* was distinctly different to that in CH and NH *E. nitens*. Large differences in foliar N and P have been reported previously between these species following fertilisation (Judd *et al.* 1996). N levels of *E. nitens* seedlings are generally lower than those of *E. globulus* (Dell *et al.* 1982;

Bennett *et al.* 1996; Knight and Nicholas 1996). At planting, total N of NH *E. globulus* was nearly 1.5 times that of NH *E. nitens*. Higher soluble and nucleic acid N in NH *E. globulus* compared to CH and NH *E. nitens* seedlings at planting may indicate greater N uptake and higher leaf metabolic activity respectively (Capin and Kedrowski 1983). Higher nucleic acid P concentrations in *E. globulus* at planting indicates high levels of RNA and protein synthesis (Brady 1973) as RNA comprises about 90% of plant nucleic acids (Bieleski 1973). These results are consistent with the higher shoot:root ratios of NH *E. globulus* at planting. Nitrogen was supplied in nitrate form in the fertiliser mix and this may suggest that *E. globulus* is more efficient at absorbing nitrate N. However, both *E. globulus* (Shedley *et al.* 1993) and *E nitens* (Garnet and Smethurst 1999) have been shown to preferentially absorb ammonium N. After acclimation to field conditions soluble and nucleic acid N fractions in *E. globulus* followed patterns similar to those of *E. nitens*. However, higher levels of insoluble P complexes in *E. globulus* throughout the experiment and higher levels of protein N in acclimated (week 9 and beyond) *E. globulus* seedlings may indicate inherent differences in foliar N and P partitioning between the two species. Higher protein N may be necessary in acclimated *E. globulus* seedlings to enable rates of photosynthesis (see Chapter 3) similar to that of *E. nitens*.

Species differences in photosynthetic cold-hardening

Increases in soluble P and labile inorganic P may be indicative of photosynthetic cold hardening (Labate and Leegood 1990; Hurry *et al.* 1993). Soluble and labile inorganic P did not increase immediately after planting in NH *E. globulus* and were consistently lower in *E. globulus* compared to *E. nitens* except at planting. This may

indicate an inherently lower capacity of *E. globulus* to acclimate to the changed photosynthetic conditions after planting relative to *E. nitens*.

Retranslocation of N and P in CH, NH E. nitens and NH E. globulus

The decrease in total foliar N concentrations between planting and week 1 in all seedling treatments may indicate N translocation from shoot to root. Shoot:root ratio is highly plastic and a function of environmental conditions (Lambers and Poorter 1992). In general, assimilates are partitioned preferentially to shoots if conditions are limiting for photosynthesis and to roots if nutrients or water are limiting. Decreases in root:shoot ratio following increased nutrient supply have been reported for *E. grandis* (Cromer and Jarvis 1990; Kirschbaum *et al.* 1992), *E. globulus* (Ericsson 1994) and *E. nitens* (Misra *et al.* 1998). Root:shoot ratios of seedlings produced in nurseries are often unusually low due to frequent water and nutrient application (Ledig 1983). As there was probably little water stress after planting, reduction of N supply from roots to shoots following transplanting may have induced changed partitioning of assimilates to roots which required N re-mobilization from shoot to root. Growth after planting of *P. glauca* seedlings was higher in nutrient-loaded compared to non-loaded seedlings and was related to the ability of seedlings to retranslocate internal nutrient reserves for new growth. Little or no net uptake of nutrients during the first season after planting was reported (McAlister and Timmer 1998). Shoots of transplanted *P. mariana* seedlings have been reported to significantly lower levels of all macronutrients, some micronutrients, amino acids and sucrose compared to non-transplanted controls (Kim *et al.* 1999). Transplanted seedlings were watered and their shoot water potential did not differ compared to control seedlings. Nutrient

translocation and shoot:root adjustment occurs in *P. mariana* and *P. glauca* seedlings after transplanting, as was observed in *Eucalyptus* seedlings investigated here.

The larger decrease of total N of NH *E. globulus* compared to *E. nitens* between planting and week 1 may reflect a greater requirement for root:shoot adjustment in *E. globulus* than *E. nitens*. This is consistent with NH *E. globulus* having a lower root:shoot compared to *E. nitens* at planting.

Soluble and nucleic acid N in CH, NH E. nitens and NH E. globulus

There was a relatively large increase in soluble N from week 1 to week 13 in all seedling treatments indicating soil N uptake. This was associated with a more gradual increase of protein N which indicates partitioning of soluble to protein N. Soluble and protein N increased in parallel to increases in total N and A_{max}. The dependent relationship of photosynthesis (and growth) on total N is well established (Field and Mooney 1986). In *Pinus sylvestris* L. accumulation of soluble N has been reported to result from yearly application of N fertiliser and was associated with increased growth (Nasholm and Ericsson 1990).

Levels of nucleic acid N found here for *Eucalyptus* seedlings and that of *Picea mariana* [Mill.] B.S.P., *Larix laricina* [Du Roi] K. Koch, *Betula papyrifera* var. humilis [Reg.] Fern, and Raup and *Alnus crispa* [Ait] Pursch seedlings (Chapin and Kedrowski 1983) were two-fold higher than those reported in 6 year-old *E. globulus* (Hooda and Weston 1999). This may be a reflection of greater metabolic activity of seedling leaves compared to mature foliage.

Nutrient relations of CH, NH and NS E. nitens

Foliar nutrient relations of CH and NH *E. nitens* were largely similar throughout the experiment. However, the observation of lower N levels in all fractions in CH *E. nitens* compared to NH *E. nitens* at planting was unexpected. Increased foliar N, in the form of photosynthetic enzymes, is a common response of many plant species to cool temperatures, including *E. nitens* (Warren 1996). Low N of CH vs NH *E. nitens* observed here may have been caused by lower potting mix temperatures induced by overnight cold-hardening limiting the uptake of N in CH *E. nitens*. Differences in N between these treatments occurred only at planting. A study of the effects of N fertiliser on *E. globulus* concluded that any foliar increases in total N were short term in six-year-old trees and had dissipated by four years after the fertiliser had been applied (Hooda and Weston 1999). In the seedlings used here, differences had dissipated in a matter of four weeks.

The rapid recovery of foliar nutrient levels of NS *E. nitens* was associated with large increases in total N and P. These increases were paralleled by considerable increase in soluble N which is indicative of soil N uptake. Total N and P increased from extremely low levels to levels similar to the other fertilised *E. nitens* treatments within 6 weeks after planting.

Conclusion

Inherent differences in nutritional physiology found here between *E. nitens* and *E. globulus* may explain the more vigorous growth of *E. globulus* in the nursery and the possible inability of *E. globulus* to adjust photosynthetically to conditions of cold-induced photoinhibition. However, it appears that the retranslocation of foliar

nutrients to roots and the root:shoot adjustment from nursery to field, is similar for both species. Following acclimation, soil N and P was acquired and all N and P fractions gradually increased in all seedling treatments. This was paralleled by increases in photosynthesis and growth. The above conclusions are based on changes within distinct chemical fractions of N and P. Analysis of total N and P only would not have led to the same conclusion.

Only after 20 weeks of establishment were total N and P levels measured here similar to those reported in other studies (Judd *et al.* 1996; Bennett *et al.* 1996). This demonstrates that rapid and dynamic changes in N and P levels occur in seedlings during establishment which are distinct from those observed in older, established eucalypts. Additionally, it appears that the pre-conditioning treatment of withholding nutrients does not detrimentally affect growth of *E. nitens* seedlings planted in spring on a mild site at 350 m asl.

Chapters 3 and 4 have reported effects of seedling hardening pre-treatment and differences between *E. globulus* and *E. nitens* on cold-induced photoinhibition, pigment chemistry and N and P nutrition physiology during establishment at 350 m asl. At this altitude the severity of cold-induced photoinhibition adversely affected growth of *E. globulus* but not *E. nitens*. Further, the crucial role of cold-induced photoinhibition and pigment chemistry was emphasised in the initial investigation of Chapter 3. For these reasons the possible effects of more severe cold-induced photoinhibition on growth and N and P nutrition physiology on *E. nitens* at 700 m asl are investigated in the following chapter. Cold-induced photoinhibition and the resulting pigment chemistry are studied in greater detail.

Chapter 5. Effects of hardening pre-treatments after early-winter planting at 700 m asl

Introduction

Light-adapted chlorophyll fluorescence

The ratio of variable to maximal chlorophyll fluorescence (F_v/F_m) is the most often reported and recommended measure of cold-induced photoinhibition in tree seedlings (Ball *et al.* 1994). This measure of maximal PSII efficiency of dark-adapted leaves correlates well with the maximum quantum yield of carbon fixation (Genty *et al.* 1989). Photochemical and non-photochemical quenching under light-stable conditions in the field can be estimated by modulated fluorometry (Bilger *et al.* 1995).

Gas exchange vs. chlorophyll fluorescence

Cold temperatures depress photosynthesis not only through active down-regulation of light utilisation but through rate limitation of enzymes of the Calvin cycle (Ottander and Öquist 1991). This limitation by the dark reactions is not assessed using chlorophyll fluorescence. If gas exchange is measured concurrently with chlorophyll fluorescence a disparity between the levels of photoinhibition indicated is observed. In *Acer platanoides* L. this led to the suggestion that upper cells of the leaf filter excess light, causing photoinhibition in this cell layer to be greater than at the whole leaf level (Schindler and Lichtenthaler 1996).

The xanthophyll cycle and chlorophyll fluorescence

Recently, a connection between decreases in photosynthetic efficiency and the active down-regulation of photosynthesis has been reported (Adams and Demmig-Adams

1994; 1995). This occurs through de-epoxidation of violaxanthin to zeaxanthin (via antheraxanthin) in response to a decrease in thylakoid lumen pH (Yamamoto 1985; Gilmore and Yamamoto 1993). Large changes in zeaxanthin levels were found to closely follow changes in incident PFD (Adams and Demmig-Adams 1992).

Ecological implications of the xanthophyll cycle

Large variations in conversion states of the xanthophyll cycle (both pre-dawn and diurnal) and pool sizes of xanthophyll-cycle pigments have been reported between sun and shade species as well as plants of the same species acclimated to different levels of light (Thayer and Björkman 1990; Oberhuber and Bauer 1991; Demmig-Adams and Adams 1992; Adams *et al.* 1994). Shading, by reducing excess light, results in smaller xanthophyll pools and lower xanthophyll:chlorophyll ratios (Adams and Demmig-Adams 1992; Logan *et al.* 1998a). Plants not under excessive light are characterised by higher levels of total chlorophyll and lower chlorophyll *a*:*b* ratios (Adams and Demmig-Adams 1992; Logan *et al.* 1998a; García-Plazaola *et al.* 1999a). Lower total chlorophyll and higher chlorophyll *a*:*b*, xanthophyll cycle pool sizes and xanthophyll:chlorophyll ratios are also found at the top compared to the bottom of an altitudinal cline (Polle *et al.* 1999) or in summer compared to winter (Adams and Barker 1998; Logan *et al.* 1998b). Evergreen tree species such as *Pinus ponderosa* (Adams and Demmig-Adams 1994) and *Eucalyptus pauciflora* (Roden *et al.* 1999) are especially good examples for demonstrating these foliar pigment adjustments.

Nutrient deprivation, through decreasing photosynthetic capacity, increases the excess of excitation energy (Jacobs 1995; Verhoeven *et al.* 1997) and decreases the ability of the plant to synthesise and repair any damage to PSII (Balachandren and Osmond

1994; Godde and Hefer 1994). Nutrient deprivation has the same effects on foliar pigments as well lit relative to shaded conditions, high relative to low altitude or winter vs summer season ie. lower total chlorophyll and higher xanthophyll cycle pool sizes (Verhoeven *et al.* 1997, Morales *et al.* 1998).

There is a view that the xanthophyll cycle accounts for all excess energy dissipation in plants (Demmig-Adams and Adams 1996). Reports investigating the correlation of chlorophyll fluorescence with xanthophyll cycle conversion states (Ottander *et al.* 1995; Shang and Feierabend 1998; Thiele *et al.* 1998) and more recent studies involving mutants implicate D1 protein turnover and PSII functional arrangement as intimately involved in the dissipation of excess light energy (Forster *et al.* 1999; Darkó *et al.* 2000). This seems to particularly be the case where cold-induced photoinhibition is severe, such as that measured in eucalypt seedlings during establishment (see Chapter 3).

Photooxidation of chlorophylls, xanthophylls and other carotenoids

Absorption of light causes excitation of chlorophyll which in turn can lead to the formation of triplet state chlorophyll (Owens 1996) and/or the triplet state chlorophyll dimer (P680) (Aro et al 1993). Triplet state chlorophyll and P680 can interact with O_2 to produce singlet oxygen (Foote 1976). Excessive light conditions increase the yield of triplet state chlorophyll and P680 and thus of singlet state oxygen (Niyogi 1999). Singlet oxygen oxidises lipid proteins and chlorophyll, xanthophyll and other carotenoid pigments in the vicinity of its generation (Knox and Dodge 1985). The leaf damage observed in photoinhibited eucalypt seedlings (Chapter 3) indicated that pigment photooxidation probably occurs in establishing eucalypt seedlings.

Hypotheses tested

It was shown in Chapter 3 that photoinhibition was the major process contributing to physiological stress during establishment of *E. globulus* and *E. nitens* seedlings on a 350 m asl site. However, the magnitude of the stress was not sufficient to affect growth of *E. nitens*. This first experiment was undertaken in a spring planting. The physiological expression of characteristics developed at sea level in the nursery was evaluated and this information used to define characteristics required for successful establishment of seedlings under the planting conditions. To provide a benchmark for the planting of *E. nitens* seedlings, an experiment, containing only this species, and combining the shading and nutrient-starving treatments of Chapter 3, was designed. Environmental conditions at the planting site at 700 m asl, were considered likely to induce severe cold-induced photoinhibition which would facilitate investigation of the possible effects of photoinhibition on growth of *E. nitens*. The experiment was established in May and seedlings raised at a high altitude nursery in order to avoid the confounding effects of acclimation following transfer from the nursery at sea level to the planting environment. Given the relative importance of effects of cold-induced photoinhibition on seedling establishment, chlorophyll fluorescence and leaf pigment chemistry were studied in greater detail than in Chapter 3.

The hypotheses postulated and tested in this experiment were;

- that non-shaded and fertilised seedlings will have lower levels of pre-dawn photochemical efficiency (F_v/F_m), light-stable chlorophyll fluorescence parameters and gas-exchange rates on diurnal and seasonal scales compared to shaded and nutrient-starved seedlings;

• that diurnal and seasonal patterns in carotenoid and chlorophyll pigments will correlate to levels of cold-induced photoinhibition measured by chlorophyll fluorescence and gas exchange and;

• that the effects of cold-induced photoinhibition will adversely affect growth of non-shaded and fertilised seedlings relative to shaded and nutrient-starved seedlings.

Materials and methods

Plant material

Seedlings were raised from Rubicon 79 3324 seedlot (North Forest Products Pty. Ltd. improved seed) in 115 cm^3 plugs in the North Forest Products' Somerset nursery which is situated at sea level. The potting mix was saturated with Aquasol®every 10 days (solution concentration 1100-1500 mS). After 8 months, seedlings were moved to a second nursery at 350 m asl for 1 month. Half the seedlings were fed twice weekly with Aquasol® (referred to as the fertilised [F] treatment) while the others were starved of nutrients (referred to as the non-fertilised [NF] treatment).

Experimental site

The 0.75 ha site was approximately 60 km south of Ridgley, Tasmania at latitude-longitude 391250 E, 5406100 N (AMG reference). The altitude was 700 m asl with a mean annual rainfall and temperature of 2400 mm and 7.9 °C, respectively. The area was surrounded by an electrified fence. The site was prepared using a Savannah mound plough as per conventional practice. No fertilisers, herbicides or insecticides were applied throughout the period of the experiment.

Experimental treatments and Layout

Seedlings were planted on 10 June 1998. Between and within row spacings were 3.5 m and 0.6 m, respectively. Half the seedlings planted were immediately surrounded by a tree shelter. The shelter consisted of 3 wooden stakes that supported 50% shadecloth to 1.8 m above ground. Seedlings were planted such that they were in groups of four consisting of one of the following treatments: Non-Shaded Non-Fertilised (NF); Non-Shaded Fertilised (F); Shaded Non-Fertilised (Sh-NF); Shaded Fertilised (Sh-F). Seedling treatments were randomly allocated within the groups of four. One hundred and four groups of seedlings were planted in a completely randomised design of three rows of 25 seedling groups and a fourth row of 29 seedling groups, which included a plot of 4 seedling groups allocated to physiological measurements. A completely randomised design was implemented on the basis of climate, slope, aspect and uniformity of soil conditions across the experimental site.

The seedling groups that form the physiology plot were selected on the basis of ease of access. The fifty groups in the inner two rows were used for pigment sampling and height measurement. The fifty groups in the outer two rows were used for destructive sampling.

Initial average seedling height was 26.3 and 27.0 cm for non-fertilised and fertlised seedlings, respectively.

Micrometeorology

Air and soil temperature, total incident shortwave radiation, relative humidity, wind speed and rainfall were monitored at the site using an automatic weather station

(Environdata, Warwick, Queensland). The quantum sensor on the chlorophyll fluorometer was used to estimate the average levels of light attenuation through the shadecloth tree shelters.

Gas exchange

The procedure used was detailed in Chapter 2. Gas-exchange measurements were made on 2, 17 and 30 June, 21 July, 6 and 31 August, 8 October, 11 November 1998 and 20 January 1999 (0, 1, 3, 6, 8, 12, 16, 20 and 32 weeks after planting respectively) between 1100 and 1400 h Australian Eastern Standard Time (AEST).

Chlorophyll fluorescence

Procedures used were detailed in Chapter 2. Pre-dawn maximal photochemical efficiency, F_v/F_m, was assessed on the same days as gas exchange measurements. Investigation of diurnal fluctuations in steady-state fluoresence parameters (yield and NPQ) was conducted on 21 May (before planting), 23 June, 13 August, 21 September, 3 November 1998 and 20 January 1999 (-2, 2, 8, 15, 19 and 32 weeks after planting respectively).

Chlorophyll and carotenoid analysis

Procedures used were detailed in Chapter 2. Sampling was conducted after chlorophyll fluorescence measurements made on 21 May (pre-planting), 23 June, 8 October 1998 and 20 January 1999 (-2, 2, 15 and 29 weeks after planting respectively).

Visible/near infrared spectroscopy (VIS-NIRS)

The procedure used for VIS-NIRS analysis was detailed in Chapter 2. Leaf material for measurement was sampled on 8 October 1998 (15 weeks after planting).

Growth Analysis

The procedure used was detailed in Chapter 2. All seedlings allocated to non-destructive measurement were measured for height. Seedlings were sampled on 4 June, 8 October and 2 December 1998 and 25 March, 8 October 1999 (0, 18, 26, 42 and 70 weeks after planting respectively).

Nutrient analysis

The procedure used was detailed in Chapter 2. Seedlings were sampled on 4 June, 8 October and 2 December 1998 and 25 March 1999 (0, 18, 26 and 42 weeks after planting respectively).

Statistical analysis

The procedures used for linear regression of NPQ vs yield, yield and NPQ vs A+Z/V+A+Z and analysis of all other data were detailed in Chapter 2.

Results

Micrometeorology

The number of frosts, absolute and average minimum, and average temperatures experienced by seedlings during the experiment are shown in Table 5.1. Micrometeorological data showed that there was no difference in minimum temperature experienced by shaded and non-shaded seedlings (see Appendix 4).

Relative humidity data (not shown) indicated that low humidity did not limit seedlings during establishment (consistently > 85 %). The primary difference between shaded and non-shaded treatments was the light environment. PAM-2000 data indicated that tree shelters shielded seedlings from 54 % of incident photosynthetically active radiation (PAR). A seedling the morning after a frost in the 3rd week after planting is shown in Plate 5.1.

Table 5.1. Number of frosts and absolute and average minimum and maximum air temperatures (°C) measured at 1.3 m during the period of physiological measurements of cold-induced photoinhibition.

Weeks after planting	Number of Frosts week^{-1}	Absolute minimum	Average minimum	Average maximum
1	1	-3.9	2.2	8.4
2	0	0.5	2.4	6.8
3	3	-4.6	-0.2	5.1
4	1	-2.4	2.0	6.8
5	4	-3.3	1.2	5.9
6	3	-3.4	0.5	7.2
7	3	-0.9	0.7	7.7
8	1	-0.3	2.6	6.7
9	3	-2.3	1.0	8.8
10	6	-5.5	-2.1	7.5
11	4	-4.3	-0.3	8.3
12	3	-3.2	0.4	10.6
16	1.8	-2.2	2.7	9.7
20	1.5	-1.1	2.9	10.3
24	2.3	-4.1	2.1	13.7
28	0.3	-0.6	5.6	15.3
30	0.5	-0.7	7.6	15.8

Plate 5.1. *E. nitens* seedling under conditions of cold-induced photoinhibition the morning after an over-night frost during the 3rd week after planting.

Chlorophyll fluorescence

F_v/F_m

Pre-dawn F_v/F_m of Sh-F seedlings was maintained at optimal levels (> 0.7) throughout the establishment period (Figure 5.1). F_v/F_m of the other treatments generally declined until 6 weeks after planting and then increased to optimal levels at 32 weeks after planting. The decline in F_v/F_m was most pronounced in the NF treatment. Minimum F_v/F_m measured was 0.16, 0.44, and 0.56 in the NF, F, and Sh-NF respectively.

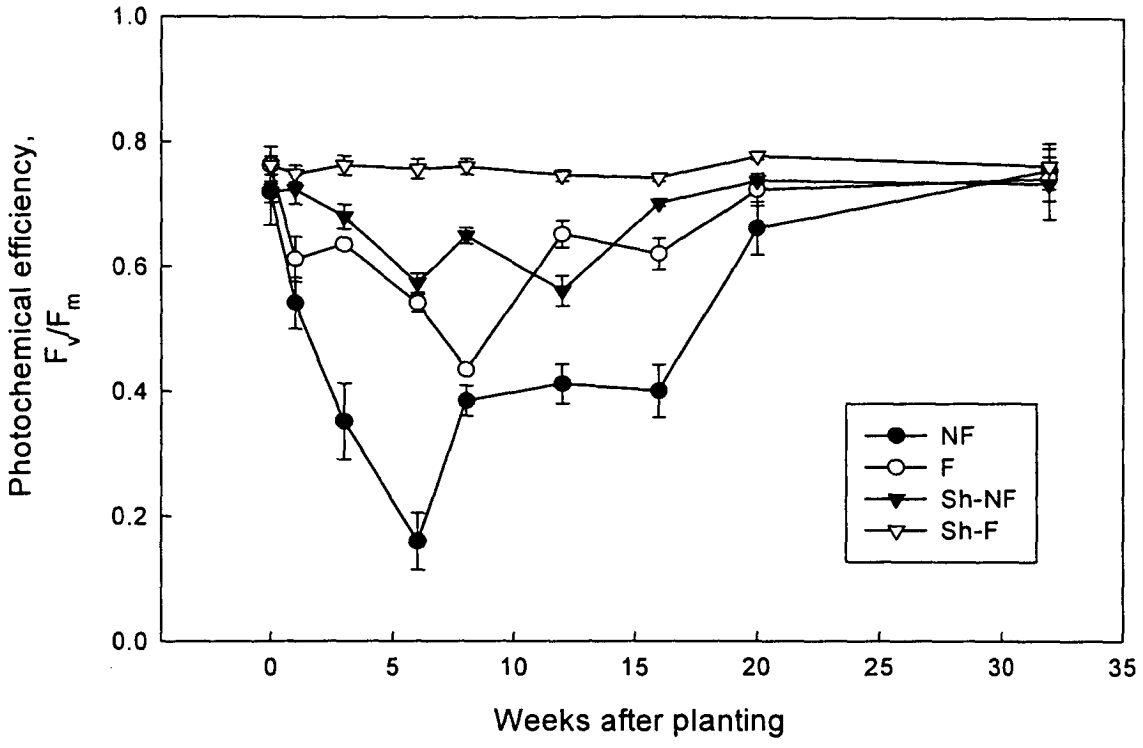


Figure 5.1. Pre-dawn photochemical efficiency (F_v/F_m; dimensionless) with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

NPQ

NPQ of all treatments was high (15-25) before planting until 15 weeks after planting after which NPQ remained low (Figure 5.2). NPQ of Sh-F seedlings decreased ($p < 0.05$) from week 8. However NPQ of F, Sh-NF and NF seedlings did not decrease ($p < 0.001$) until week 15 at which time Sh-F had further decreased ($p < 0.001$). NPQ of non-fertilised seedlings was significantly higher ($p < 0.05$) than fertilised seedlings before planting. NPQ of Sh-F seedlings was significantly lower ($p < 0.005$) than that of other treatments at 15 weeks after planting. Temperatures at approximately 0800 h were 12.8, 2.1, 3.7, 9.0, 13.8 and 14.7 °C for 0, 2, 8, 15, 18 and 32 weeks after planting, respectively. The possibility of undetected errors (causing the very high

NPQ values) in the use of the PAM-2000 was eliminated as described in the discussion. Appendix 5 contains a subset of raw data and NPQ calculation.

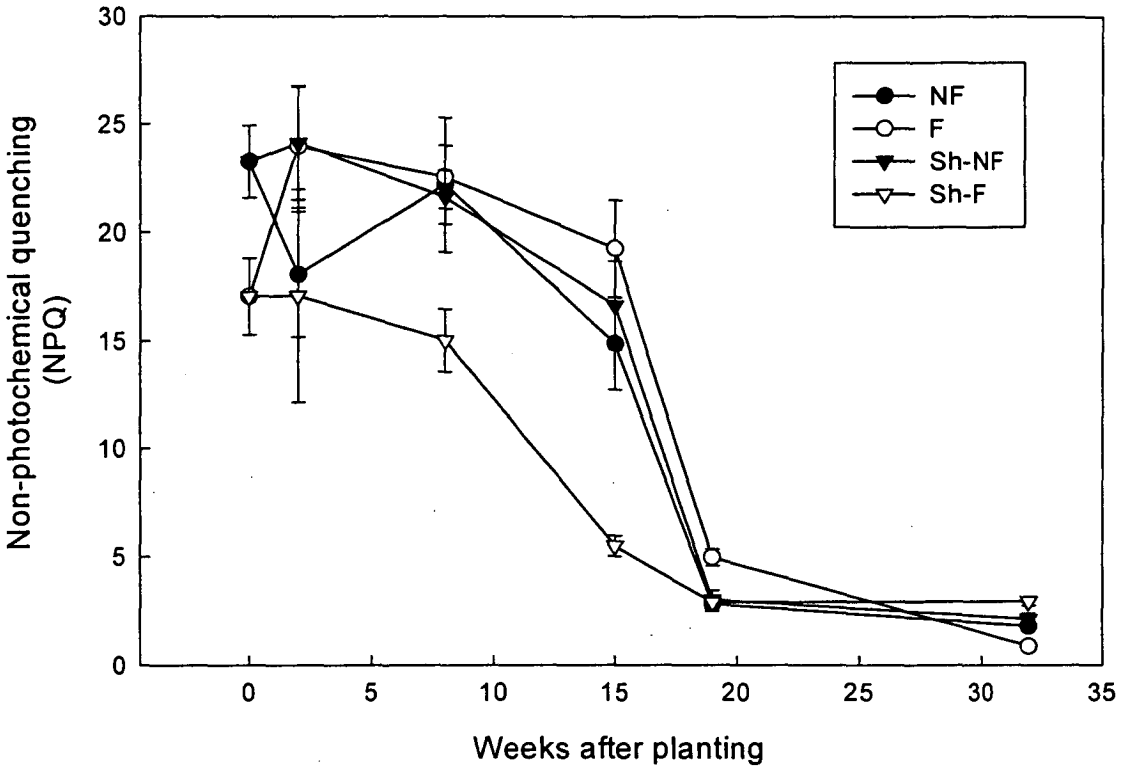


Figure 5.2. Maximal non-photochemical quenching (NPQ; dimensionless) with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

NPQ vs Yield

Linear regression analysis revealed that variation in NPQ with yield was similar between treatments before planting (Table 5.2). However, the slope and intercept of the regression of NF differed to that of F, Sh-NF and Sh-F seedlings 2 and 9 weeks after planting indicating that for a given NPQ, yield of NF seedlings was lower

relative to the other treatments. By 14 and 20 weeks after planting regression of NPQ vs yield was the same for all treatments. At 32 weeks after planting, the slope and intercept of the regression indicated that F seedlings were developing less NPQ relative to yield than other treatments, although this difference was small compared to differences observed between treatments just after planting. The slopes of regressions decreased with time in concert with the general decrease of NPQ (Figure 5.2).

Table 5.2. Slope and r^2 values of linear regression of NPQ vs. yield with time after planting. Effects of intercept were small compared to slope but were significantly different between treatments whenever slopes differed. Differences were at least $p < 0.01$.

	NPQ vs. Yield		
Week	Treatment	Slope	r^2
0	All	-31.4	0.93
2	NF	-102.9	0.88
	F, Sh-NF and Sh-F	-42.0	0.75
9	NF	-37.6	0.65
	F, Sh-NF and Sh-F	-25.4	0.67
14	All	-17.9	0.47
20	All	-4.2	0.25
32	NF, Sh-NF and Sh-F	-3.0	0.80
	F	-2.1	0.70

Gas Exchange

A_{max} of all treatments generally decreased from planting until 6 weeks after planting and then increased to reach optimal levels ($A_{max} > 15$ μmol m^{-2} s^{-1}) by 32 weeks after

planting (Figure 5.3). A_{max} of fertilised was greater ($p < 0.0001$) than that of non-fertilised treatments before planting. One and three weeks after planting shaded had higher ($p < 0.0001$) A_{max} than the corresponding non-shaded treatments. At 6, 8, and 12 weeks after planting A_{max} was similar between NF and Sh-NF seedlings. A_{max} in F was higher than that of NF and Sh-NF ($p < 0.001$) seedlings and Sh-F higher than that of F ($p < 0.001$) seedlings. A_{max} was Sh-F > Sh-NF = F > NF ($p < 0.005$) 16 weeks after planting but was not significantly different between treatments thereafter.

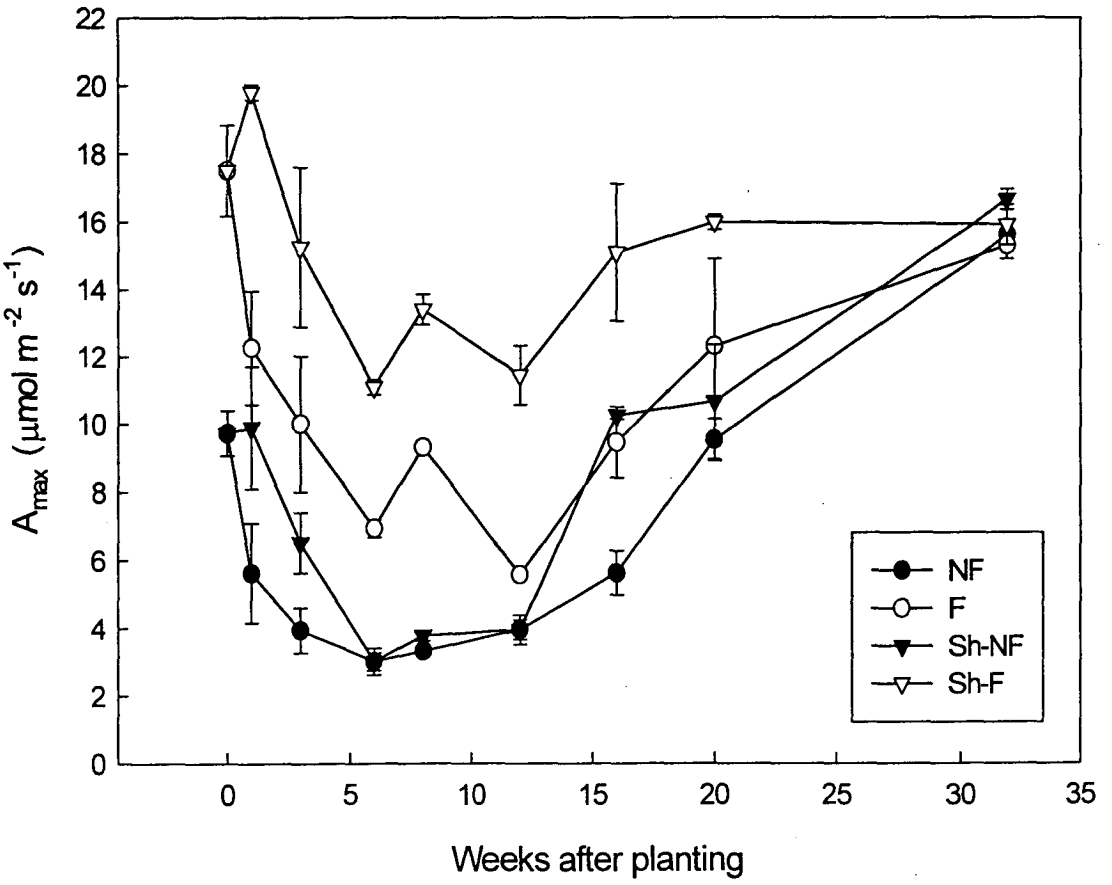


Figure 5.3. Maximum photosynthesis (A_{max}; µmol m^{-2} s^{-1}) with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Pigments

Pigment composition: differences within non-fertilised and fertilised treatments

Foliar total chlorophyll content decreased significantly between planting and 2 weeks after planting in NF ($p < 0.05$) and F ($p < 0.05$) (Tables 5.3a and 5.3b) but remained constant in Sh-NF and Sh-F seedlings (Tables 5.3c and 5.3d). However, total chlorophyll of Sh-NF and Sh-F decreased ($p < 0.005$ and ns) between 2 and 9 weeks after planting. Total chlorophyll increased between 9 and 32 weeks after planting in NF ($p < 0.001$) and Sh-NF (ns) but decreased in F (ns) and Sh-F ($p < 0.001$) seedlings. Chlorophyll *a*:*b* ratios followed the same trends as total chlorophyll except ratios had decreased by 2, and not 9 weeks, after planting in shaded treatments. By 9 weeks after planting chlorophyll *a*:*b* ratios of shaded treatments had returned to pre-planting levels. Increases in the ratio were not significant between 9 and 32 weeks for NF seedlings.

Patterns of change in carotenoid levels per unit chlorophyll were similar between NF and Sh-NF seedlings. Compared to before planting, neoxanthin and lutein had increased at 2 and 9 weeks after planting in NF (ns, $p < 0.05$) and Sh-NF ($p < 0.01$, ns) and then decreased from 9 to 32 weeks after planting in NF ($p < 0.0001$, 0.001) and Sh-NF ($p < 0.05$, 0.001) seedlings. In contrast V+A+Z decreased from before planting to 2 and 9 weeks after planting in NF (ns) and Sh-NF ($p < 0.05$) and then increased from 9 to 32 weeks in NF (ns) and Sh-NF ($p < 0.01$) seedlings. On a leaf area basis, neoxanthin, lutein and β-carotene were relatively constant before planting and 2 and 9 weeks after planting in NF and Sh-NF but increased by 32 weeks after planting in NF ($p < 0.05$, 0.05, 0.0001) and Sh-NF ($p < 0.0001$, 0.0001, 0.001)

seedlings. V+A+Z did not significantly differ in F or Sh-F except between weeks 9 and 32 in Sh-F seedlings when V+A+Z decreased ($p < 0.005$).

Patterns of change in carotenoid levels expressed per unit chlorophyll were similar between F and Sh-F treatments. Compared to before planting neoxanthin had increased at 2 and 9 weeks after planting in F ($p < 0.0001$) and Sh-F ($p < 0.01$) and decreased between 9 weeks to 32 weeks after planting in F (ns) and Sh-F ($p < 0.005$) seedlings. No trends with time were apparent in lutein or V+A+Z. On a leaf area basis, neoxanthin, lutein and β-carotene were relatively constant in F and Sh-F until a general decrease by 32 weeks after planting in F ($p < 0.05$, 0.05, ns) and Sh-F ($p <$ 0.0001, 0.0001, 0.001) seedlings. V+A+Z remained relatively constant in F but decreased ($p < 0.001$) between 9 and 32 weeks after planting in Sh-F seedlings.

Pigment compositions: differences between non-fertilised and fertilised treatments

Before planting and 2 and 9 weeks after planting, total chlorophyll and chlorophyll *a*:*b* were higher ($p < 0.001$, 0.05) in F and Sh-F compared to NF and Sh-NF seedlings. Throughout the experiment on a per unit chlorophyll basis, lutein, and particularly V+A+Z, were significantly lower in F and Sh-F relative to NF and Sh-NF ($p < 0.01$, 0.05) seedlings whereas neoxanthin and β-carotene were similar. However, on a leaf area basis, neoxanthin, lutein, V+A+Z and β-carotene were all greater in F and Sh-F compared to NF and Sh-F seedlings except at planting when V+A+Z was similar between NF and F seedlings. At 32 weeks after planting levels of all chlorophylls and carotenoids were relatively similar between treatments compared to differences observed at other measurement dates.

Table 5.3. Total chlorophyll content (μmol m^{-2}), chlorophyll *a:b* ratio (dimensionless) and carotenoids (μmol m^{-2} or mmol mol^{-1} total $chlorophyll^{-1}$) of (a) NF, (b) F, (c) Sh-NF and (d) Sh-F *E. nitens* seedlings sampled 0, 2, 19 and 32 weeks after planting.

(a)	**NF**			
	Weeks after planting			
	0	2	9	32
Total chlorophyll (μmol m^{-2})	212 ± 15	180 ± 18	178 ± 15	321 ± 19
Chl *a:b*	3.76 ± 0.49	3.34 ± 0.63	3.54 ± 0.53	3.87 ± 0.53
Carotenoids (mmol mol^{-1} total $chlorophyll^{-2}$)				
Neoxanthin	49 ± 1	54 ± 1	55 ± 1	46 ± 2
Lutein	226 ± 2	251 ± 6	257 ± 5	219 ± 5
V+A+Z	271 ± 5	180 ± 3	167 ± 3	212 ± 7
β-Carotene	89 ± 2	85 ± 2	84 ± 2	96 ± 4
Carotenoids (μmol m^{-2})				
Neoxanthin	10 ± 5	9 ± 9	9 ± 5	15 ± 5
Lutein	48 ± 11	45 ± 9	45 ± 26	70 ± 15
V+A+Z	57 ± 5	32 ± 3	29 ± 3	68 ± 7
β-Carotene	18 ± 2	15 ± 3	15 ± 2	31 ± 4

(b)	**F**			
	Weeks after planting			
	0	2	9	32
Total chlorophyll (μmol m^{-2})	393 ± 31	354 ± 11	386 ± 106	263 ± 24
Chl *a:b*	3.90 ± 0.58	3.60 ± 0.21	3.83 ± 1.83	4.01 ± 0.61
Carotenoids (mmol mol^{-1} total $chlorophyll^{-2}$)				
Neoxanthin	48 ± 6	54 ± 3	54 ± 18	47 ± 6
Lutein	196 ± 21	211 ± 7	219 ± 67	218 ± 14
V+A+Z	138 ± 16	164 ± 10	129 ± 26	203 ± 13
β-Carotene	88 ± 14	87 ± 6	85 ± 29	87 ± 10
Carotenoids (μmol m^{-2})				
Neoxanthin	19 ± 2	19 ± 1	21 ± 7	12 ± 2
Lutein	77 ± 8	75 ± 3	84 ± 26	57 ± 4
V+A+Z	54 ± 6	58 ± 4	50 ± 10	53 ± 3
β-Carotene	34 ± 5	31 ± 2	32 ± 11	22 ± 3

(c)	Sh-NF			
	Weeks after planting			
	0	2	9	32
Total chlorophyll (μmol m^{-2})	212 ± 15	213 ± 12	169 ± 15	267 ± 18
Chl *a*:*b*	3.76 ± 0.49	3.33 ± 0.41	3.79 ± 0.65	3.80 ± 0.46
Carotenoids (mmol mol^{-1} total chlorophyll^{-2})				
Neoxanthin	49 ± 5	53 ± 5	60 ± 7	47 ± 6
Lutein	226 ± 11	231 ± 15	299 ± 27	196 ± 23
V+A+Z	271 ± 22	149 ± 11	210 ± 20	127 ± 9
β-Carotene	89 ± 11	86 ± 9	77 ± 8	79 ± 7
Carotenoids (μmol m^{-2})				
Neoxanthin	10 ± 1	11 ± 1	10 ± 1	12 ± 2
Lutein	48 ± 2	49 ± 3	50 ± 5	52 ± 6
V+A+Z	57 ± 5	31 ± 2	35 ± 3	34 ± 3
β-Carotene	18 ± 2	18 ± 2	13 ± 1	21 ± 2

(d)	Sh-F			
	Weeks after planting			
	0	2	9	32
Total chlorophyll (μmol m^{-2})	393 ± 31	397 ± 24	305 ± 31	263 ± 29
Chl *a*:*b*	3.90 ± 0.58	3.61 ± 0.47	3.85 ± 0.58	3.85 ± 0.78
Carotenoids (mmol mol^{-1} total chlorophyll^{-2})				
Neoxanthin	48 ± 6	52 ± 4	65 ± 9	44 ± 9
Lutein	196 ± 21	193 ± 25	274 ± 21	200 ± 37
V+A+Z	138 ± 16	116 ± 7	256 ± 60	129 ± 19
β-Carotene	88 ± 13	90 ± 8	88 ± 6	77 ± 13
Carotenoids (μmol m^{-2})				
Neoxanthin	19 ± 2	20 ± 2	20 ± 3	11 ± 2
Lutein	77 ± 8	77 ± 10	83 ± 7	52 ± 10
V+A+Z	54 ± 7	46 ± 3	78 ± 18	33 ± 5
β-Carotene	34 ± 5	36 ± 3	27 ± 2	20 ± 3

Pre-dawn A+Z/V+A+Z

A+Z/V+A+Z ratio of non-fertilised treatments was high before planting and remained relatively constant throughout the experimental period except in Sh-NF seedlings which decreased ($p < 0.0001$) between 9 to 32 weeks after planting (Figure 5.4). The ratio in fertilised treatments was relatively low before planting but had increased at 2 weeks after planting. A+Z/V+A+Z then remained high for F but decreased ($p < 0.0001$) for Sh-F seedlings between 9 and 32 weeks. Before planting A+Z/V+A+Z of non-fertilised was higher than that of fertilised seedlings ($p < 0.05$). At 2 weeks after planting A+Z/V+A+Z of NF remained higher ($p < 0.01$) than that of F seedlings. Levels were similar between treatments at 9 weeks after planting but were higher in non-shaded ($p < 0.001$) compared to shaded treatments at 32 weeks after planting.

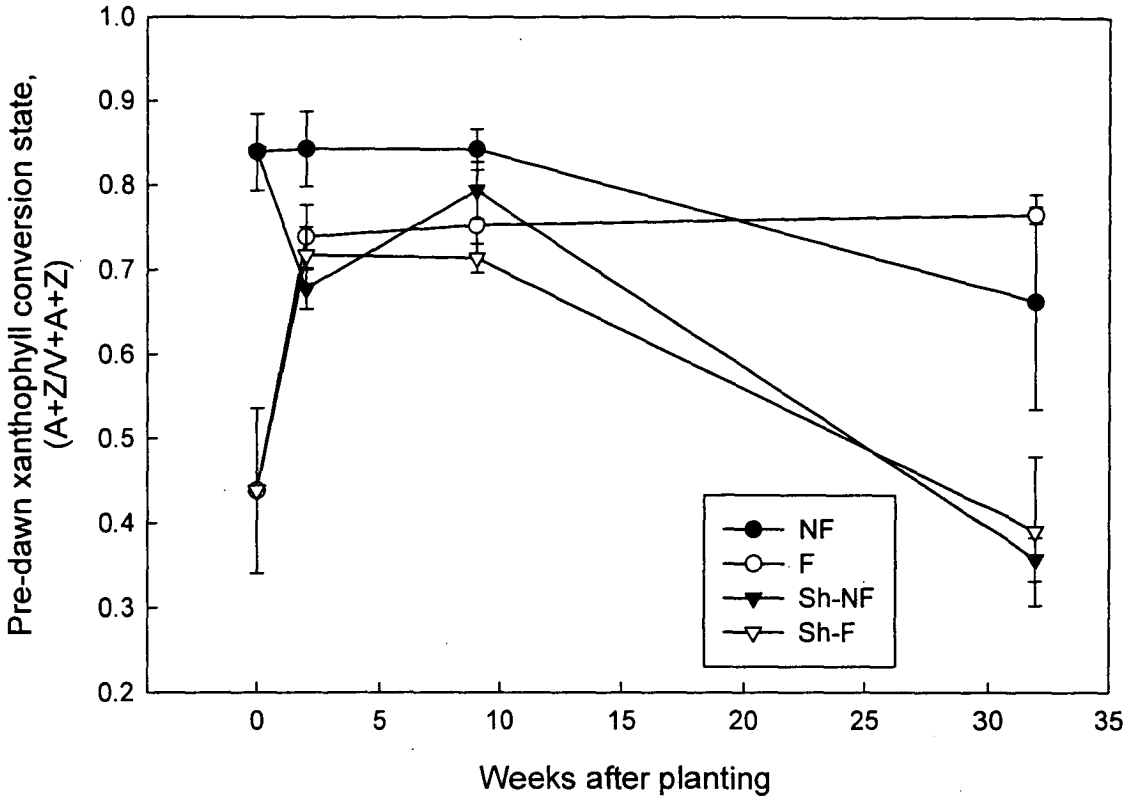


Figure 5.4. Xanthophyll-cycle conversion state (A+Z/V+A+Z; dimensionless) with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Pigment:chlorophyll fluorescence comparisons

Diurnal variations in NPQ, A+Z and PAR

Diurnal variation of NPQ was similar to that of incident PAR on all measurement dates (Figures 5.5a, c, e and g) and at all times during the day except at 1100 and 0900 h before planting (Figure 5.5a) and 2 weeks after planting (Figure 5.5b), respectively, when PAR was high relative to NPQ. In contrast A+Z/V+A+Z showed less diurnal variation (Figures 5.5b, d, f and h).

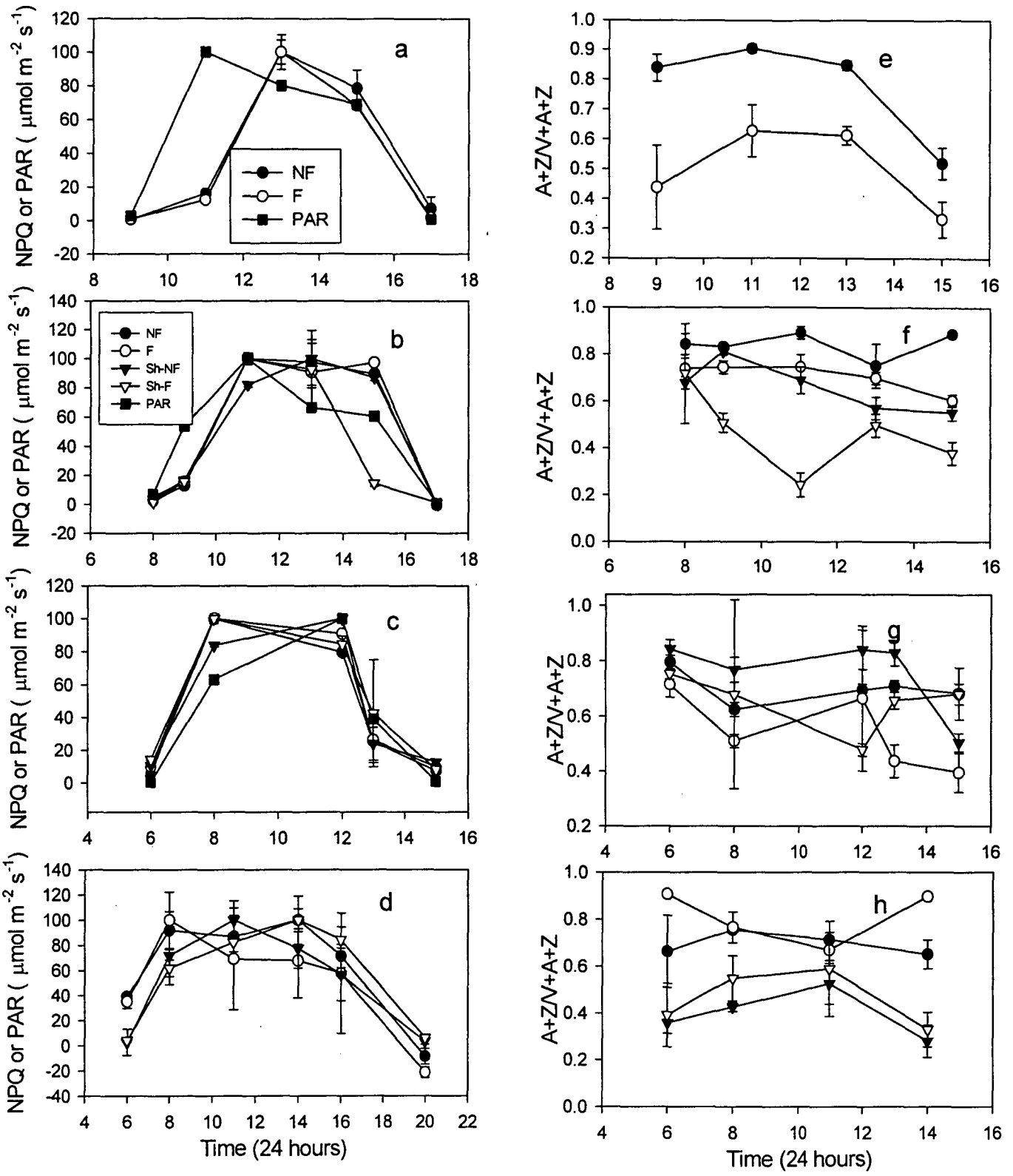


Figure 5.5. Diurnal variation (a, b, c and d) of non-photochemical quenching (NPQ; dimensionless) and incident photosynthetically active radiation (PAR; µmol m^{-2} s^{-1}) (expressed as % of maximum) and (e, f, g and h) xanthophyll cycle conversion state (A+Z/V+A+Z; dimensionless), with time after planting for NF, F, Sh-NF and Sh-F *E.*

Yield vs A+Z/V+A+Z

Linear regression analysis showed that yield increased with decreasing A+Z/V+A+Z before planting and was similar between non-fertilised and fertilised treatments (Table 5.4a). However, no relationship was found at 2 or 9 weeks after planting. At 32 weeks after planting a linear relationship between yield and A+Z/V+A+Z was observed in all treatments. The relationship was similar between Sh-NF and Sh-F seedlings, and the slopes indicated a significantly lower ($p < 0.01$) A+Z/V+A+Z for a given yield relative to NF or F seedlings.

NPQ vs A+Z/V+A+Z

NPQ increased with A+Z/V+A+Z and was similar between NF and F before planting (Table 5.4b). No relationship was observed between NPQ and A+Z/V+A+Z at 2 or 9 weeks after planting. 32 weeks after planting the slope of NPQ vs A+Z/V+A+Z was similar between NF, Sh-F and Sh-NF seedlings. Y intercepts differed in the order of magnitude of Sh-NF > Sh-F > NF ($p < 0.01$). No relationship was observed in F seedlings.

Table 5.4. Slope and r^2 values of linear regression of (a), yield and (b), NPQ vs. xanthophyll cycle conversion state (A+Z/V+A+Z) with time after planting. Effects of intercept were small compared to slope but significantly different between treatments whenever slopes differed.

(a)	**Yield vs. A+Z/V+A+Z**		
Week	Treatment	Slope	r^2
0	All	-1.0	0.60
2	No relationship		
9	No relationship		
32	NF	-6.9	0.86
	F	2.7	0.85
	Sh-NF, Sh-F	-2.6	0.76

(b)	NPQ vs. A+Z/V+A+Z		
Week	Treatment	Slope	r^2
0	All	31.2	0.52
2	No relationship		
9	No relationship		
32	NF	7.8	0.71
	F	No relationship	
	Sh-NF	11.5	0.62
	Sh-F	10.9	0.95

Nutrient analysis

Total foliar N

Total N decreased between planting and 15 weeks after planting in NF and Sh-NF ($p < 0.0001$) and F and Sh-F seedlings ($p < 0.001$) (Figure 5.6a). From 15 to 38 weeks

after planting total N levels steadily increased ($p < 0.05$) in all treatments. At planting and 15 weeks after planting total N was higher in fertilised compared to non-fertilised treatments ($p < 0.0001$, 0.05, respectively).

Nucleic acid N

Nucleic acid N decreased between planting and 15 weeks after planting in NF and Sh-NF ($p < 0.01$) and F and Sh-F ($p < 0.005$) seedlings (Figure 5.6b). Nucleic acid N increased ($p < 0.01$) in all treatments between 15 to 38 weeks after planting. At planting nucleic acid of non-fertilised treatments was significantly lower ($p < 0.01$) than that of fertilised treatments.

Soluble (nitrate, ammonia and amino acid) N

Soluble N decreased ($p < 0.01$) in F and Sh-F seedlings between planting and 15 weeks after planting (Figure 5.6c). Levels increased ($p < 0.05$) in all treatments between 15 to 38 weeks after planting. At planting soluble N of fertilised treatments was significantly higher ($p < 0.05$) than that of non-fertilised treatments.

Protein N

Protein N comprised > 85 % of total N (Figure 5.6d) and followed the same patterns of change as that of total N.

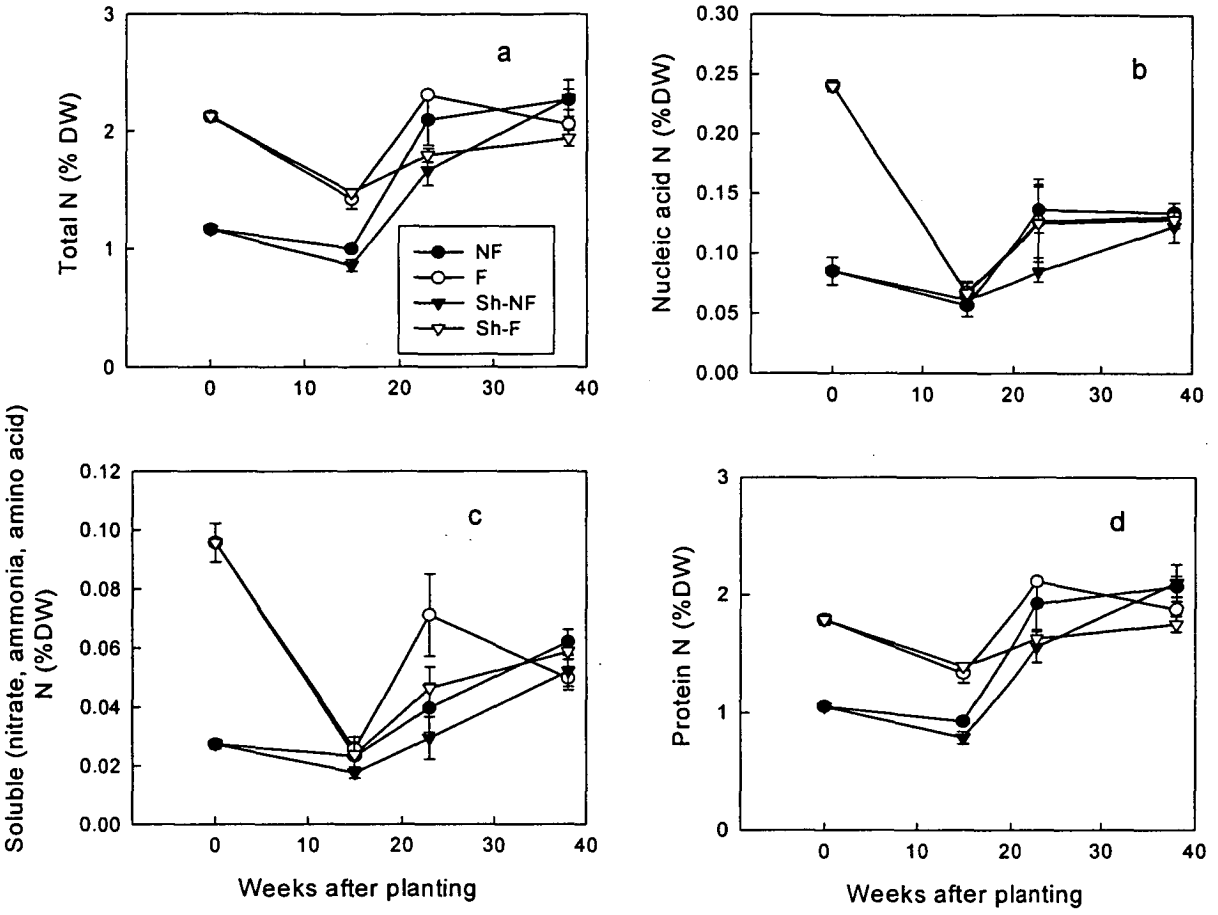


Figure 5.6. Changes in (a) total, (b) nucleic acid, (c) soluble (nitrate, ammonia and amino acid) and (d) protein nitrogen (all % dry weight), with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Total P

Total P decreased between planting and 15 weeks after planting in F and Sh-F ($p < 0.0001$) and NF and Sh-NF ($p < 0.001$) seedlings (Figure 5.7a). There were no changes in total P levels in any treatments after week 15. At planting, total P of fertilised treatments was significantly higher ($p < 0.0001$) than that of non-fertilised treatments.

Nucleic acid P

Nucleic acid P levels decreased in F and Sh-F ($p < 0.05$) and NF and Sh-NF ($p < 0.005$) seedlings between planting and 15 weeks after planting (Figure 5.7b). Levels in NF and F seedlings increased ($p < 0.0001$, 0.05 respectively) between 15 and 23 weeks after planting after which levels in NF seedlings decreased ($p < 0.05$) to 38 weeks after planting. Nucleic acid P of Sh-F and Sh-NF seedlings increased ($p < 0.05$) between 15 and 38 weeks after planting. At planting nucleic acid P of non-fertilised treatments was significantly less ($p < 0.01$) than that of fertilised treatments.

Sugar phosphate

Organic P levels decreased in F and Sh-F ($p < 0.005$) and NF and Sh-NF ($p < 0.05$) seedlings, between 0 and 15 weeks after planting (Figure 5.7c). There were no differences in organic P between 15 and 38 weeks after planting. At planting organic P was higher in fertilised ($p < 0.01$) compared to non-fertilised treatments.

Inorganic P (P_i)

P_i levels decreased in F and Sh-F ($p < 0.01$) and NF and Sh-NF ($p < 0.05$) seedlings between 0 to 15 weeks after planting (Figure 5.7d). P_i of NF, F and Sh-NF seedlings increased ($p < 0.05$) between 13 and 23 weeks after planting. P_i of F decreased significantly ($p < 0.01$) between 23 to 38 weeks after planting. P_i of Sh-F did not change between 15 and 38 weeks after planting.

Insoluble P complexes

Residual P levels decreased for F and Sh-F seedlings between planting and 38 weeks after planting ($p < 0.05$) but remained similar throughout the experimental period in NF and Sh-NF seedlings (Figure 5.7e). Residual P was higher in fertilised treatments compared to non-fertilised treatments ($p < 0.05$) before planting.

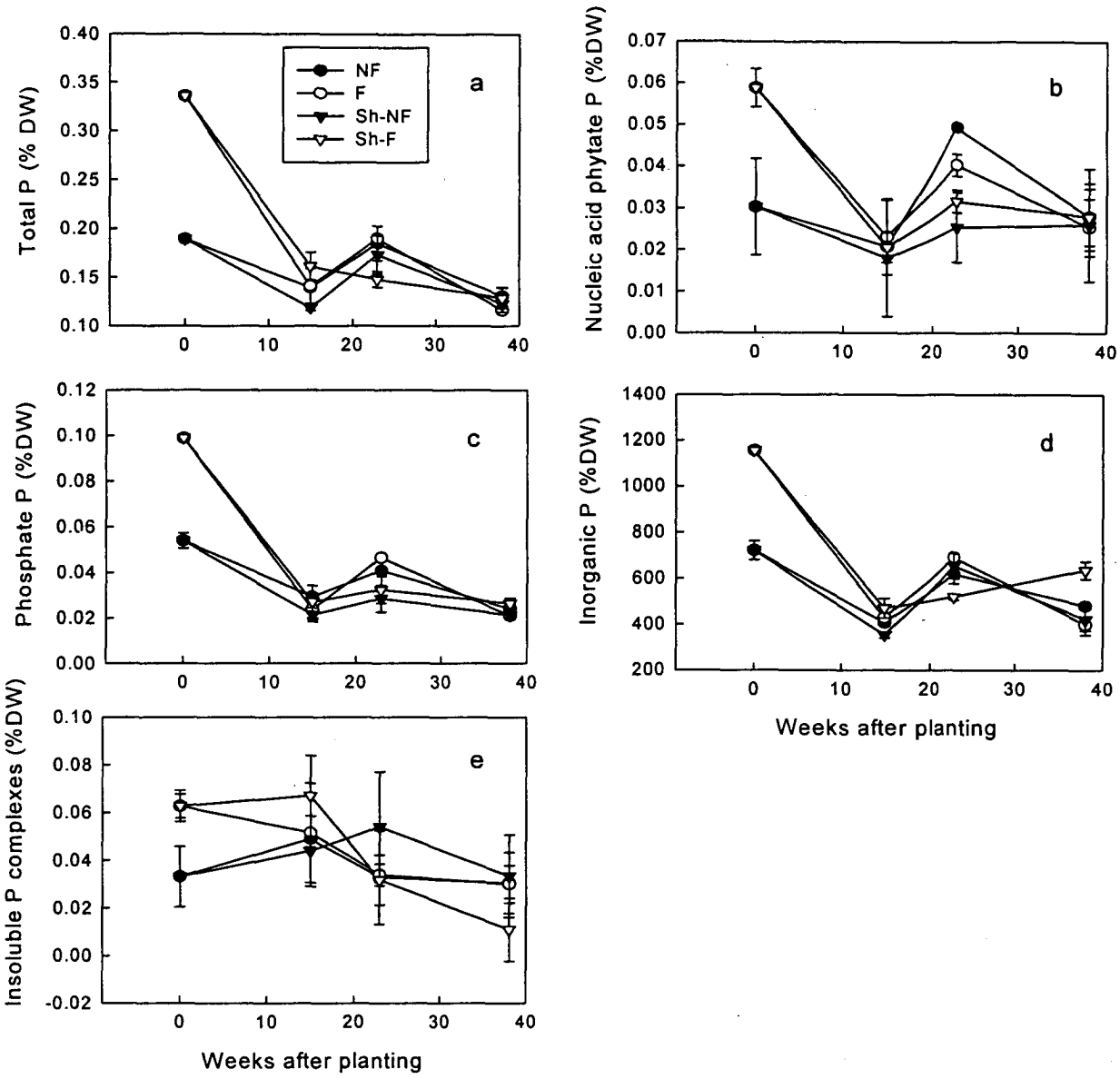


Figure 5.7. Changes in (a) total, (b) nucleic acid, (c) phosphate and (d) inorganic phosphorus and (e) insoluble P complexes (all % dry weight) with time after planting for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Growth analysis

Relative growth rate (RGR)

RGR increased between 0-18 and 18-26 weeks after planting in NF ($p < 0.0001$) and F ($p < 0.07$) seedlings (Figure 5.8a). RGR in all treatments increased between 18-26 and 26-42 weeks then decreased between 26-42 and 42-70 weeks. RGR of F was higher than that of NF seedlings ($p < 0.05$) at 0-18 weeks, while RGR of NF was higher than that of Sh-NF ($p < 0.05$) and Sh-F ($p < 0.05$) seedlings at 18-26 and 26-42 weeks, respectively.

Net assimilation rate (NAR)

NAR increased between 0-18 and 18-26 weeks after planting in NF ($p < 0.0001$) and F ($p < 0.01$) seedlings (Figure 5.8b). NAR of all treatments increased between 18-26 and 26-42 weeks and decreased between 26-42 and 42-70 weeks. NAR was higher in F compared to NF seedlings ($p < 0.05$) at 0-18 weeks. At 18-26 weeks NAR of NF was higher ($p < 0.05$) than that of Sh-NF seedlings. At 26-42 weeks NAR of NF and F was higher ($p < 0.05$) compared to that of Sh-NF seedlings.

Leaf area ratio (LAR)

LAR of all treatments decreased, increased, then decreased between 0-18 to 18-26, 18-26 to 26-42, and 26-42 to 42-70 weeks after planting, respectively (Figure 5.8c). LAR of Sh-NF seedlings was significantly higher ($p < 0.05$) than that of other treatments at 26-42 and 42-70 weeks.

Specific leaf area (SLA)

SLA of all treatments decreased, increased, then decreased between 0-18 to 18-26, 18-26 to 26-42 and 26-42 to 42-70 weeks after planting respectively (Figure 5.8d). SLA was significantly higher in F compared to NF ($p < 0.01$) and Sh-NF compared to NF, F, and Sh-F ($p < 0.01$) seedlings at 0 and 42 weeks, respectively.

Leaf:weight ratio (LWR)

LWR of all treatments decreased, increased, then decreased between 0-18 to 18-26, 18-26 to 26-42 and 26-42 to 42-70 weeks after planting, respectively (Figure 5.8e). LWR was significantly higher ($p < 0.001$) in fertilised compared to non-fertilised treatments at planting.

Root:shoot ratio

Root:shoot ratio of all treatments decreased, increased, then decreased between 0-18 to 18-26, 18-26 to 26-42 and 26-42 to 42-70 weeks after planting respectively (Figure 5.8f). Non-fertilised seedlings had higher root:shoot than fertilised ($p < 0.005$) seedlings at planting. Root:shoot ratio was F > NF > Sh-NF > Sh-F ($p < 0.05$, 0.0001 and 0.0001 respectively) at 70 weeks.

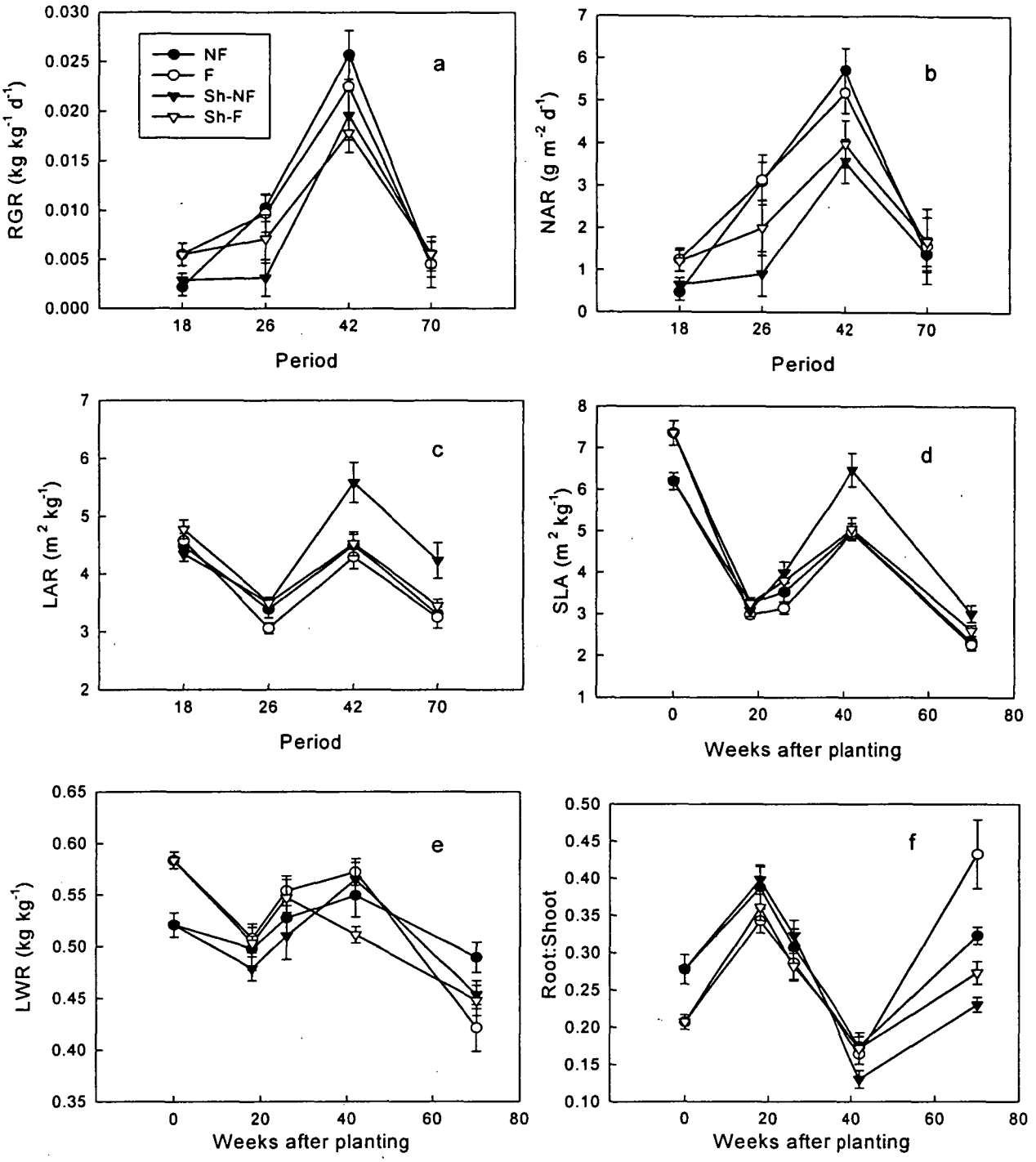


Figure 5.8. Growth analysis variables: (a) relative growth rate (RGR), (b) net assimilation rate (NAR), (c) leaf area ratio (LAR), (d) specific leaf area (SLA), (e) leaf:weight ratio (LWR) and (f) root:shoot ratio of NF, F, Sh-NF and Sh-F *E. nitens* seedlings harvested 0, 18, 26, 42 and 70 weeks after planting. Bars indicate ± standard error.

Seedling Height

Seedling height growth was not positive until 18 weeks after planting for Sh-F and 26 weeks after planting for other treatments (Figure 5.9). From week 26 all treatments increased height rapidly. At 18 and 26 weeks after planting, fertilised had attained greater height ($p < 0.0001$) than non-fertilised treatments. At 26 weeks after planting Sh-F had greater height ($p < 0.0001$) than F seedlings. At 34 weeks after planting Sh-F and Sh-NF attained greater height than F and NF, respectively. This trend continued at 42 ($p < 0.0001$) and 70 ($p < 0.005$) weeks after planting although Sh-NF was higher than F seedlings at 42 weeks after planting.

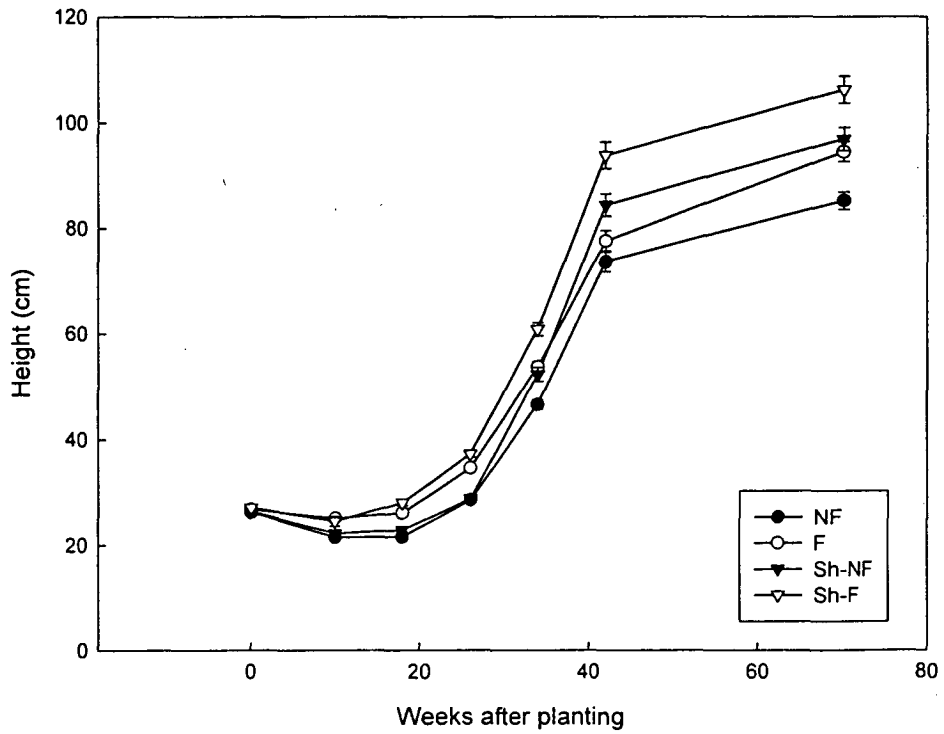


Figure 5.9. Height profile (cm) of NF, F, Sh-NF and Sh-F *E. nitens* seedlings with time after planting. Bars indicate ± standard error.

Discussion

Major findings

Seedling growth was related to light saturated levels of carbon assimilation but not chlorophyll fluorescence. The results from the chlorophyll fluorescence measurements indicate the possibility of a light filtering role of upper leaf cells. Levels of NPQ reported here are higher than previously reported. This was consistent with the high levels of photooxidation of chlorophylls and also of xanthophylls and ß-carotene in severely photoinhibited treatments. Corresponding increases in lutein and neoxanthin indicated an antioxidant role for these xanthophylls. The lack of correlation between chlorophyll fluorescence and xanthophyll cycle conversion status indicates that mechanisms other than the xanthophyll cycle may dissipate excess light energy under conditions of photoinhibition. Seedling relative growth rate (RGR) was maximised by pre-planting fertilisation during conditions of cold-induced photoinhibition, and by complete exposure (ie. non-shading) in the absence of photoinhibition. However, surrounding seedlings by shadecloth tree shelters maximises seedling height.

Seedling photosynthesis, photoinhibition and pigment chemistry

F_v/F_m vs. A_{max}

In general, with the exception of F_v/F_m of Sh-F seedlings, patterns of F_v/F_m and A_{max} were associated with seasonal changes in temperature as reported previously for a number of plant species, including *Eucalyptus* species (Farage and Long 1991; Oberhuber and Bauer 1991; Skillman *et al.* 1996; Lundmark *et al.* 1998; Schaberg *et al.* 1998; Roden *et al.* 1999). However, in relative terms, seasonal changes between treatments in F_v/F_m and A_{max} were dissimilar. Gas exchange sums the activity of the

light and dark reactions in absolute terms whereas chlorophyll fluorescence measures the potential efficiency of photosystem II of the chlorophylls present, regardless of chlorophyll level. Thus the different seasonal patterns of F_v/F_m and A_{max} were due to a combination of a) the effects of total pigment levels induced by fertiliser and shading treatments and b) the effects of environmental conditions on the dark reactions of photosynthesis.

Treatment effects

Before planting, when conditions were not likely to cause photoinhibition, F_v/F_m was high in all treatments (> 0.7, Demmig and Björkman 1987) whereas A_{max} differed significantly between fertiliser treatments and was related to foliar N and pigment levels. Thus, differences in foliar pigment levels resulted in A_{max} typical of non-photoinhibited *E. nitens* (A_{max} >15 μm m^{-2} s^{-1}, Battaglia *et al.* 1996) in fertilised treatments and indicative of nutrient stress in non-fertilised treatments. In contrast, at 1 week after planting, A_{max} of shaded treatments increased slightly while that of non-shaded treatments decreased relative to levels before planting. This indicates a down-regulation of photosynthesis caused by exposure to high light.

Effects of air temperature

After the first week of the experiment, a combination of relatively lower average minimum and/or maximum temperatures until week 12 after planting indicated that (in the light of the following studies referred to) damage to, and rates of, enzymes of dark reactions of photosynthesis may be limiting photosynthesis. Damage to dark reaction enzymes has been purported to occur after a frost of – 4.6 °C in *E. nitens* seedlings (Davidson *et al.* 1995) and dark reaction enzyme limitation in *E. nitens*

purported to occur at daytime temperatures of < 10 °C (Battaglia et al 1996). The decrease in A_{max} of shaded treatments during this period is consistent with this conclusion. Low absolute and average temperatures in the same period led to extended conditions of cold-induced photoinhibition. These were indicated by increased depression of F_v/F_m in all treatments except Sh-F compared to levels at week 1. The recovery of both A_{max} and F_v/F_m to pre-planting levels after week 12 paralleled increasing average temperatures. In Sh-F seedlings, F_v/F_m remained constant and optimal throughout the experimental period. This was consistent with the relatively high A_{max} and low NPQ levels which indicated high levels of light energy utilisation and low levels of excess energy dissipation, respectively.

Cellular layers and photosynthesis

A_{max} levels, in contrast to F_v/F_m, of NF and Sh-NF seedlings were similar at 6, 8 and 12 weeks after planting. This may point to a departure in the correspondence between the results gathered by gas exchange and those by chlorophyll fluorescence. In a similar study, relatively high A_{max} corresponded to relatively low F_v/F_m in *Quercus ilex* L. leaves during winter (García-Plazaola *et al.* 1999b). Cells in the upper layers of leaves determine F_v/F_m whereas all cells within the leaf contribute to CO_2 fixation (Day and Vogelmann 1995; Schindler and Lichtenthaler 1996). Epidermal and upper palisade mesophyll cells act as a light filter for lower palisade and spongy mesophyll cells within the leaf (Sun et al 1998). Thus saturating irradiance on sun leaves of *Spinacia oleracea* has been shown to have no effect on CO_2 fixation but to decrease F_v/F_m by 15% (Sun et al 1996). In addition, CO_2 fixation does not decrease exponentially across the leaf whereas light gradients do. In *Spinacia oleracea* the spongy mesophyll contributed 40% of carboxylation (Nishio *et al.* 1993). Thus F_v/F_m

may overestimate levels of photoinhibition of the whole leaf and this calls for concurrent measurement of gas exchange in studies of photoinhibition (Schindler and Lichtenthaler 1996). High levels of sub-epidermal anthocyanin which may have attenuated levels of absorbed radiation in NF seedlings (Krol *et al.* 1995; Barker *et al.* 1997; Dodd *et al.* 1998; Chapters 7 and 8) may have further contributed to the overestimation of photoinhibition by F_v/F_m.

NPQ

Maximum non-photochemical quenching (NPQ) levels were 5-fold higher than that previously reported for *E. nitens* seedlings (Hovenden and Warren 1998; Warren *et al.* 1998) until 19 weeks after planting (early summer). (To exclude the possibility of undetected errors in the use of the PAM-2000, on 25 September 1998 diurnal NPQ was measured on naturally regenerating *E. globulus* seedlings of similar physiological age, growing in Hobart at 30 m asl. NPQ was typical of those reported in the literature, reaching a maximum of ~ 2.7.) The clear, cloud free days occurred only after overnight frosts. Relatively high NPQ levels have been reported under such conditions if followed by frosty mornings (Spunda at al 1997; Barker *et al.* 1998). This is probably associated with post-frost depression of the dark reactions of photosynthesis (Ottander and Öquist 1991; Davidson *et al.* 1995; Battaglia *et al.* 1996). Other factors such as the low pigment levels and photosynthetic rates of young foliage (Krause *et al.* 1995; Dodd *et al.* 1998; Thiele *et al.* 1998) and close ground proximity exposing seedlings to cold air stratification (Jordan and Smith 1995) may also have contributed to the high levels of NPQ. Comparable and even lower levels of F_v/F_m (~ 0.2) have been reported in conifer seedlings exposed to severe cold-induced photoinhibition (Oberhuber and Bauer 1991; Adams *et al.* 1994; Neuner *et al.*

1999), though these were not associated with high NPQ values like those observed in *E. nitens*. Under such high levels of photoinhibition, NPQ can theoretically reach a value of 100 (Buschmann 1999).

Seasonal pigment composition

Total chlorophyll

Relatively low total chlorophyll content is a common strategy adopted by plants to balance energy absorption with energy utilisation and dissipation in high light environments (Wise 1995; Tardy *et al.* 1998). Low total chlorophyll levels are realised via photooxidation of chlorophylls in response to rapid environmental changes which lead to cold-induced photoinhibition (Haldimann *et al.* 1995; Haldimann 1996; Matoo *et al.* 1999). In the non-shaded NF and F treatments, chlorophyll levels significantly decreased (this is interpreted and referred to as photooxidisation and corroborated by decreased chlorophyll *a:b*) in the first 2 weeks after planting in response to the changed environmental conditions between the nursery and the field. This was accompanied by significant reductions in F_v/F_m. No photooxidation of total chlorophyll was observed in shaded treatments at this time though this did occur between 2 and 9 weeks after planting (not significant in Sh-F). This was accompanied by a significant decrease of F_v/F_m in Sh-NF but not Sh-F seedlings. Thus, light was probably in excess, even in shaded seedlings, during the cool conditions experienced between 2 and 9 weeks after planting. *Zea mays* grown at 14 °C compared to 24 °C, or of chilling sensitive compared to chilling tolerant genotypes, exhibited similar decreased chlorophyll levels over a 5 to 6 and 2 week period, respectively (Haldimann 1998, 1999).

Between 9 and 32 weeks after planting total chlorophylls increased in NF and Sh-NF seedlings but decreased in F and Sh-F seedlings: at 32 weeks, levels were similar between all treatments. N uptake in all chemical fractions in NF and Sh-NF seedlings between 9 and 32 weeks after planting (Figure 5.7) probably facilitated this increase. In contrast, decreased total chlorophylls in F and Sh-F seedlings may have occurred due to a reduced N supply following transplanting relative to the high N-supply in the nursery. Thus, soluble (nitrate, ammonia and amino acid) N was significantly less at 32 weeks after planting compared to pre-planting levels in these fertilised treatments.

Trends in chlorophyll *a*:*b* largely mirrored that of total chlorophyll (similar to Chapter 3). This was entirely consistent with the role of chlorophyll *a* in light absorption and high chlorophyll *a*:*b* ratios in sun relative to shade leaves (Demmig-Adams and Adams 1992).

Xanthophyll cycle

Increased pool size of the xanthophyll cycle components violaxanthin, antheraxanthin and zeaxanthin (V, A and Z respectively) per unit chlorophyll in response to increased levels of photoinhibition is a common response in plant species (Adams and Demmig-Adams 1994; 1995; Adams *et al.* 1995; Ottander *et al.* 1996; Verhoeven *et al.* 1996; Adams and Barker 1998; Logan *et al.* 1998a). Minimum F_v/F_m levels indicated that V+A+Z per unit chlorophyll should increase in the order NF > F > Sh-NF > Sh-F between the measurements before and 2 weeks after planting. However, the order was F > Sh-F > NF > Sh-NF. Thus in F seedlings, photooxidation of chlorophyll has decreased light absorption and, increased V+A+Z has increased non-radiative dissipation of absorbed light energy, to counter the effects of photoinhibition. In the

absence of excess light, Sh-F seedlings required no change in V+A+Z levels (or total chlorophyll levels) following planting.

Investigations of nutrient-starved plants have reported increased V+A+Z pools in response to increased levels of photoinhibition (Verhoeven *et al.* 1997; Morales *et al.* 1998). However, photooxidation of V+A+Z pigments has been reported in biochemical assays of antioxidant capacity of V+A+Z pigments under strongly oxidising conditions (Barry *et al.* 1990). Severely decreased V+A+Z in NF and Sh-NF seedlings after planting may indicate that photooxidation of xanthophyll cycle carotenoids, which can also function as antioxidants (Larson 1988), may have occurred in non-fertilised seedlings.

V+A+Z of Sh-NF and Sh-F seedlings increased between 2 and 9 weeks after planting during a period when conditions were unfavourable for photosynthesis. The decreased levels of incident irradiance and decreased light absorption because of photooxidation in the Sh-NF seedlings, may have prevented photooxidation of the xanthophylls in these shaded relative to the non-shaded treatments. In contrast, V+A+Z of non-shaded seedlings were photooxidised between 2 and 9 weeks after planting.

Between 9 and 32 weeks after planting conditions became favourable for photosynthesis and soil nutrient acquisition. By 32 weeks after planting V+A+Z had increased significantly in non-shaded and decreased in shaded treatments. The relatively high levels of V+A+Z of NF and F seedlings at this time may be indicative of those required in non-shaded seedlings for the dissipation of all excess light energy

under optimal conditions for photosynthesis and when no photooxidation of xanthophylls is occurring. In the shaded treatments, the relatively lower V+A+Z levels allowed a greater proportion of light utilisation and, as a consequence, a lesser level of excess energy was required to be dissipated. These results are consistent with the harmless dissipation of excess excitation energy that occurs in plants under optimum conditions by way of their photoprotective systems which, when overloaded, leads to photooxidation of components of the thylakoid membrane (Pallett and Young 1993).

ß-carotene

ß-carotene levels decreased in parallel with levels of V+A+Z. The sensitivity to photooxidation of ß-carotene under conditions of photoinhibition has been well documented (Satoh 1970; Mathis and Kleo1973; Telfer *et al.* 1991). It has been proposed that oxidation of ß-carotene destabilises the structure of PSII and triggers degradation of the D1 protein (Sandmann *et al.* 1993; Trebst and Depka 1997). Oxidisation of ß-carotene is consistent with decreases of V+A+Z being associated with photooxidation and not the harmless dissipation of light energy via xanthophyll cycling. In this study, when V+A+Z increased, ß-carotene levels did not decrease. This was particularily the case in non-shaded treatments between 9 and 32 weeks after planting where no photooxidation occurred under conditions optimal for photosynthesis.

Lutein and neoxanthin

Patterns of change with time of lutein and neoxanthin differed from that of V+A+Z and ß-carotene. Levels generally increased from between planting and 2 weeks after

planting and decreased between 9 and 32 weeks after planting. The quantitative relationships that determine how the various carotenoids respond to photoinhibition are not fully established. Some reports indicate increases of xanthophyll-cycle carotenoids only after events causing photoinhibition (Demmig-Adams and Adams 1992; Grace and Logan 1996; Tardy *et al.* 1998), others that lutein levels increase also, and concurrently with a decrease in levels of ß-carotene (Schmid and Shäfer 1994; Haldimann 1998; Logan *et al.* 1998b). Lutein has been shown to be more stable relative to other carotenoids under conditions conducive to their oxidation (Pallett and Young 1993). It has also been proposed that lutein is an effective quencher of triplet state chlorophyll, which is released during chlorosis, and can react with molecular oxygen to form toxic singlet oxygen (Adamska 1997; Munné-Bosch and Alegre 2000). In this study, lutein was higher in non-fertilised than fertilised treatments during periods of cold-induced photoinhibition but similar between treatments at 32 weeks after planting when conditions were optimal for photosynthesis.

Recently neoxanthin has also been suggested to quench triplet chlorophyll and singlet oxygen (García-Plazaola *et al.* 1999) although neoxanthin is more susceptibile to photooxidising conditions relative to xanthophyll carotenoids (Pallett and Young 1993).

Alternative forms of quenching

It has been suggested that xanthophyll-cycle mediated energy quenching accounts for all increases in thermal energy dissipation in higher plants (Demmig-Adams and Adams 1996). This assertation was based on close relationships between changes in

intrinsic PSII efficiency (F_v/F_m), non-photochemical quenching (NPQ) and levels of antheraxanthin and zeaxanthin (A+Z/V+A+Z) in leaves which were independent of species or conditions causing the photoinhibition (Demmig-Adams and Adams 1996). In the current study, both diurnally (yield and NPQ) and seasonally (F_v/F_m), variation in fluorescence parameters did not match variation in A+Z/V+A+Z. This effect was particularly evident between 2 and 9 weeks after planting when no diurnal relationship existed between either yield or NPQ and A+Z/V+A+Z. Even when significant relationships existed between these variables, at planting and 32 weeks after planting, diurnal variation of A+Z/V+A+Z was far less than that of the fluorescence parameters or compared to variation in A+Z/V+A+Z reported elsewhere (Adams and Demmig-Adams 1992; 1994; Barker and Adams 1997; Logan *et al.* 1997; Adams and Barker 1998; Thiele *et al.* 1998; Schiefthaler *et al.* 1999). These results may indicate that the xanthophyll cycle was operating to capacity at less than full sunlight [(A+Z/V+A+Z) > 0.8] in *E. nitens* seedlings. Thus as light increased, the conversion state could not increase further and other mechanisms contributed to diurnally increasing NPQ. A considerable volume of recent evidence suggests that reorganisation of light harvesting complexes (Ottander *et al.* 1995; Giardi *et al.* 1996, 1997; Geiken *et al.* 1998; Matoo *et al.* 1999) and particularly altered D1 protein metabolism (Thiele *et al.* 1996; Shang and Feirabend 1998; Forster *et al.* 1999; Darkó *et al.* 2000) may provide alternative forms of excess light energy quenching. However, a limitation of these results exists in that A+Z/V+A+Z is assessed from across the leaf depth whereas fluorescence measurement is restricted to upper cellular layers. Relationships between F_v/F_m and NPQ and A+Z/V+A+Z may therefore be closer if it were possible to measure chlorophyll fluorescence across the whole leaf depth.

Seedling Nutrition

Nitrogen

The decrease of total N after planting in all seedling treatments was analogous to that after planting reported in Chapter 4 and to other investigations following transplanting of tree seedlings (McAlister and Timmer 1998; Kim *et al.* 1999). It is likely that N was remobilised and translocated from shoot to root due to the reduction of supply of N from the roots after removal from the nursery environment. This supports new root growth and re-balances the root:shoot ratio to make it more suitable for the field environment (Rietveld 1989). In support of this conclusion, greater relative changes in root:shoot ratio and reduction of foliar N were observed in fertilised compared to non-fertilised seedlings.

Levels of nucleic acid and soluble N were similar between fertilised and non-fertilised treatments at 15 weeks after planting whereas there were large differences in total N. This difference was mirrored by similar differences in protein N and was consistent with the higher chlorophyll levels observed in fertilised treatments as the majority of foliar N is partitioned to support photochemical processes (Skillman *et al.* 1996). Similar and low levels of soluble and nucleic acid N indicate low soil N uptake and low metabolic activity, respectively. This may be expected given the cold conditions experienced during this period. In contrast, N uptake and metabolic activity were higher, particularly in fertilised treatments, before planting and during the warm conditions of the early summer 15 weeks after planting.

Phosphorus

In concert with total N, total P decreased significantly immediately after planting, and analogous to the changes in total N, P may have been re-mobilised and translocated from shoot to root to re-balance root:shoot ratio (Ledig 1983). Re-translocation of P can be inferred from differences between P content of old and new foliage (Munson and Timmer 1990; Folk and Grossnickle 2000). Thus changes in total P were greater in fertilised than non-fertilised seedlings after planting as the potential for re-translocation of P depends on the extent of P available in previously formed tissues (Folk and Grossnickle 2000). In conifer seedlings, up to 75 % of P requirements of new tissue is achieved by internal translocation, primarily from needles produced in the previous year (Turner and Singer 1976; van den Driessche 1985).

Seedling growth

Height

Until 26 weeks after planting, levels of A_{max} were related to seedling height growth ie. Sh-F > F > Sh-NF = NF. This demonstrates that increase in initial seedling height at the start of the experiment was greater in seedlings with high foliar nutrient status irrespective of shading.

Subsequently, environmental conditions were favourable for plant growth, photosynthetic rates were high, photoinhibition minimal and foliar N content increased due to soil uptake. Height growth of Sh-NF accelerated beyond that of NF seedlings and surpassed that of F seedlings. Height growth of Sh-F increased also above that of F seedlings. In the absence of photoinhibition, the superior height growth of shaded treatments may have been due to enhanced apical dominance

(Beetson *et al.* 1991). Results from the growth analysis are consistent with this as the relative growth rate of shaded seedlings was less than that of non-shaded seedlings. However, if early seedling height is desired, for example to escape vertebrate browsing or cold air stratification, tree shelters which promote apical dominance, may be of practical benefit.

Growth analysis

The ranking of RGR for the 0-18 week period after planting of Sh-F = F > Sh-NF = NF was consistent with that of height growth during the same period. However, in contrast to height growth, NF and F seedlings exhibited a higher RGR throughout the 18-26 and 26-42 week period after planting. Thus, in the absence of cold-induced photoinhibition, non-shaded seedlings had greater RGR compared to shaded seedlings. In a comparison using shadecloth tree shelters that eliminated a range of levels of incident light, 50 % shelters were found to provide a better compromise between protection from photoinhibiton during winter and absorption of sufficient light for height growth during spring than 70 % shelters (Holly *et al.* 1994).

Changes in NAR paralleled those of RGR. Variation in RGR during establishment of *Eucalyptus pauciflora* under conditions of low temperature has similarly been found to be positively correlated with NAR (Ball *et al.* 1997).

Differences in NAR between treatments indicated by growth analysis were consistent with instantaneous NAR indicated by A_{max} for the 0-18 week period after planting but opposite to that in the 18-26 week period. Presumably the shadecloth decreased NAR

due to decreased light absorption by seedlings within the shelters, while maximum photosynthetic rates remained unaffected.

Conclusions

Fertilising seedlings maximised early growth on a 700 m asl site planted in late autumn. Factors which contributed to this were; a) higher foliar pigment levels that allowed higher rates of photosynthesis and; b) greater nutrient reserves that were available for new root growth. This conclusion is at odds with the current industry practice of planting nutrient-starved seedlings on cold sites. Shade-cloth tree shelters were not of benefit for biomass accumulation over entire growing seasons but tree shelters of some form that provide side shade may be useful if greater seedling height is desired. Gas exchange was superior to chlorophyll fluorescence for predicting current seedling growth. However, gas exchange assessment is considerably less rapid and practical than chlorophyll fluorescence for large-scale screening of seedlings.

Investigation of diurnal F_v/F_m variation early in this experiment revealed that F_o and F_m, the components of F_v/F_m, were not relaxing after conventional (15-30 min) or extended (2-3 h) dark adaptation periods. This indicated the possibility of sustained photosynthetic downregulation in *E. nitens* foliage. This is the subject of investigation in the following chapter.

Chapter 6. Sustained Xanthophyll Cycle Engagement is an overwintering strategy of *E. nitens* seedlings

Introduction

Ecological implications of sustained xanthophyll cycle engagement

Photoinhibition occurs as a necessary process during diurnal exposure to high light (Demmig-Adams and Adams 1996; Demmig-Adams *et al.* 1996; Gilmore 1997). However, additional stress factors, in particular cold winter temperatures, lead to sustained xanthophyll cycle-dependent energy dissipation and nocturnal retention of Z and A (Adams *et al.* 1994; Adams and Demmig-Adams 1995; Verhoeven *et al.* 1996; Adams and Barker 1998; Barker *et al.* 1998). This strategy of downregulating photosynthesis has been postulated to protect leaves from photodamage, particularly during early morning following exposure to severe winter conditions overnight (Ottander and Öquist 1991; Huner *et al.* 1993; Ottander *et al.* 1995).

Mechanisms of relaxation from sustained xanthophyll cycle engagement

Two distinct forms of recovery kinetics from sustained xanthophyll cycle-dependent energy dissipation have been observed but each is not necessarily expressed in a single species. The first is rapid recovery of F_v/F_m and epoxidation of Z+A to V upon warming. The second involves slow recovery of F_v/F_m and epoxidation of Z+A to V upon warming (Verhoeven *et al.* 1998).

The mechanisms that underpin sustained engagement of the xanthophyll cycle are not accurately defined. Rapid recovery of F_v/F_m upon warming is associated with recovery from low lumenal pH (Gilmore and Björkman 1994, 1995). ATP-dependent

reverse proton transport (requiring high ATP/ADP foliar ratios) may effect and maintain low lumenal pH at low temperatures, maintaining high levels of Z+A (Gilmore and Björkman 1995). The mechanism of slow recovery of F_v/F_m and Z and A to V conversion is less well understood. Using *Pinus ponderosa* (slow recovery) and *Malva neglecta* (rapid recovery) as test species, Verhoeven *et al.* (1999) explored different cold acclimation strategies. During winter *P. ponderosa* had a decreased photosynthetic capacity, persistent reduction of F_v/F_m and retention of high Z+A, and no change in (relatively low) foliar ATP/ADP ratios. In contrast, *M. neglecta* had increased rates of photosynthesis, no change in carotenoid:chlorophyll ratio, and persistent reduction of F_v/F_m and retention of high levels of Z and A associated with high ATP/ADP ratios during only the coldest nights. Recovery of foliage under warm, low light conditions was largely complete in 15 mins in *M. neglecta*. Complete recovery took 5 and 100 h in *M. neglecta* and *P. ponderosa* respectively. Recovery of F_v/F_m was related to foliar ATP/ADP ratios in *M. neglecta*, consistent with the purported mechanism of high ATP/ADP ratios maintaining low lumenal pH (Gilmore and Björkman 1994, 1995). The slow recovery of F_v/F_m and persistent engagement of Z and A in *P. ponderosa* may be linked to re-organisation of the light-harvesting complex through protein synthesis (Ottander 1995).

Hypotheses tested

The results from Chapter 5 suggest that sustained nocturnal engagement of the xanthophyll cycle occured in *E. nitens* seedlings planted in early winter. This conclusion was based on; 1) persistent reductions of pre-dawn F_v/F_m; 2) relaxation of F_v/F_m not occurring in dark-adapted leaves over a period of several hours during attempts to ascertain variation in diurnal F_v/F_m in the field; and 3) analysis of

xanthophyll cycle conversion indicating nocturnal retention of Z and A. Two hypotheses are proposed and tested;

- that sustained levels of low F_v/F_m and high xanthophyll cycle conversion states (A+Z/V+A+Z) will relax after exposure of leaves to artificial warm, low light conditions and;
- that relaxation kinetics of F_v/F_m and A+Z/V+A+Z will be related to one another.

Materials and methods

Environmental monitoring during seedling sampling

Environmental monitoring at the Moory Rd. trial site was detailed in Chapter 2.

Plant material and sustained xanthophyll cycle engagement and relaxation

Four whole seedlings (including roots) of each of NF, F, Sh-NF and Sh-F treatments were randomly selected and sampled at approximately midday on 28 October 1998 from the Moory Rd trial (see Chapter 5). They were kept well watered, shielded from light and transported to the laboratory at Hobart. The seedlings were kept in the dark at approximately 21 °C overnight. On 29 October at 1200 h F_v/F_m of all seedlings was assessed. The seedlings were labelled and transferred to a cool room where they were placed in a completely randomised configuration and exposed to PFD of approximately 280 μmol m^{-2} s^{-1} under two metal halide lamps (see Appendix 6 for spectral quality) for 3 h at 5 °C. NPQ was measured at the start of each hour of the treatment. Leaf temperatures were approximately 12 °C. Smaller seedlings were elevated to ensure uniform exposure to light.

After exposure, the first pair of fully expanded leaves of each seedling was excised and immediately transferred to moist filter paper in covered petri dishes. These leaves were exposed to an incident PFD of approximately 10 μmol m^{-2} s^{-1} at 21 °C. F_v/F_m, NPQ and pigment sampling were conducted on one leaf of each pair during the period of recovery of the xanthophyll cycle.

Chlorophyll fluorescence, pigment sampling and analysis

The procedures followed were detailed in Chapter 2 (after Adams *et al.* 1994).

Statistical analysis

Differences in reported variables were analysed as detailed in Chapter 2.

Results

Pigment composition: differences within non-fertilised and fertilised treatments

Total chlorophyll content of foliage sampled on 28 October 1998 was similar between NF and Sh-NF but significantly lower ($p < 0.01$) in F compared to Sh-F seedlings (Table 6.1). Per unit chlorophyll, levels of lutein ($p < 0.0005$) and xanthophyll cycle components (V+A+Z) ($p < 0.01$) were higher in NF relative to Sh-NF seedlings whereas, levels of neoxanthin and β-carotene were similar: there were no differences between levels of carotenoids per unit leaf area. Per unit chlorophyll, F had greater levels of lutein ($p < 0.005$) and V+A+Z ($p < 0.05$) compared to Sh-F seedlings, whereas neoxanthin and β-carotene levels were similar. F had lower levels of all carotenoids relative to Sh-F seedlings per unit leaf area.

Table 6.1. The total chlorophyll content (μmol m^{-2}), the chlorophyll *a*:*b* ratio (dimensionless) and carotenoids (the latter expressed in μmol m^{-2} and mmol mol^{-1} total $chlorophyll^{-1}$) of NF, F, Sh-NF and Sh-F *E. nitens* seedlings during recovery of leaves under artificial warm, low light conditions.

	NF	F	Sh-NF	Sh-F
Total chlorophyll (μmol m^{-2})	154 ± 21	222 ± 14	214 ± 1	546 ± 24
Chl *a*:*b*	3.98 ± 0.92	3.88 ± 0.40	3.86 ± 0.05	3.86 ± 0.34
Carotenoids (mmol mol^{-1} total $chlorophyll^{-2}$)				
Neoxanthin	54 ± 7	51 ± 1	49 ± 1	50 ± 2
Lutein	364 ± 56	265 ± 37	246 ± 1	203 ± 6
V+A+Z	197 ± 32	149 ± 13	122 ± 9	108 ± 6
β-Carotene	93 ± 15	86 ± 6	82 ± 1	84 ± 6
Carotenoids (μmol m^{-2})				
Neoxanthin	8 ± 1	11 ± 1	10 ± 1	27 ± 1
Lutein	56 ± 9	59 ± 9	53 ± 1	111 ± 3
V+A+Z	30 ± 5	33 ± 3	26 ± 2	59 ± 3
β-Carotene	14 ± 2	19 ± 1	17 ± 1	46 ± 3

Pigment composition: Differences between non-fertilised and fertilised treatments

Total chlorophyll content was higher in F and Sh-F compared to NF ($p < 0.05$) and Sh-NF ($p < 0.01$) seedlings, respectively (Table 6.1). Chl a:b ratios did not differ between treatments. Per unit chlorophyll, levels of lutein ($p < 0.05$) and V+A+Z ($p < 0.05$) were higher in non-fertilised than fertilised treatments. Levels of neoxanthin and β-carotene were similar between treatments. Per unit leaf area NF and F seedlings had similar levels of neoxanthin, lutein, V+A+Z and β-carotene. In contrast, neoxanthin, lutein, V+A+Z and β-carotene were lower ($p < 0.01$, 0.005, 0.01 and 0.01 respectively) in Sh-NF compared to Sh-F seedlings.

Environmental monitoring

For the month prior to sampling, seedlings were exposed to average minimum and maximum temperatures of 0.4 and 10.6 °C with an absolute minimum of – 3.4 °C (Table 5.1 in Chapter 5).

Effects of artificial cold-induced photoinhibition

Assessment of non-photochemical quenching (NPQ) during conditions of artificial cold-induced photoinhibition (results not shown) indicated that leaves were exposed to excess light. Extremely high levels of NPQ (20 – 42) were recorded.

Relaxation of PSII efficiency (F_v/F_m)

After 24 h of dark adaptation at warm temperature, F_v/F_m was optimal in Sh-F (0.76) and Sh-NF (0.70) seedlings: F and NF seedlings had reduced F_v/F_m levels (0.58 and 0.51 respectively) (Figure 6.1). Cold-induced photoinhibition significantly decreased F_v/F_m of all treatments in order of magnitude of NF > F > Sh-NF > Sh-F. The time required for recovery of F_v/F_m was ranked Sh-F > Sh-NF > F > NF. Total recovery times determined on the basis of no significant change in F_v/F_m between two measurements were NF - 140 h, F - 90 h, Sh-NF - 42 h and Sh-F - 17.5 h. For the NF treatment F_v/F_m had still not reached optimal levels after 142 h.

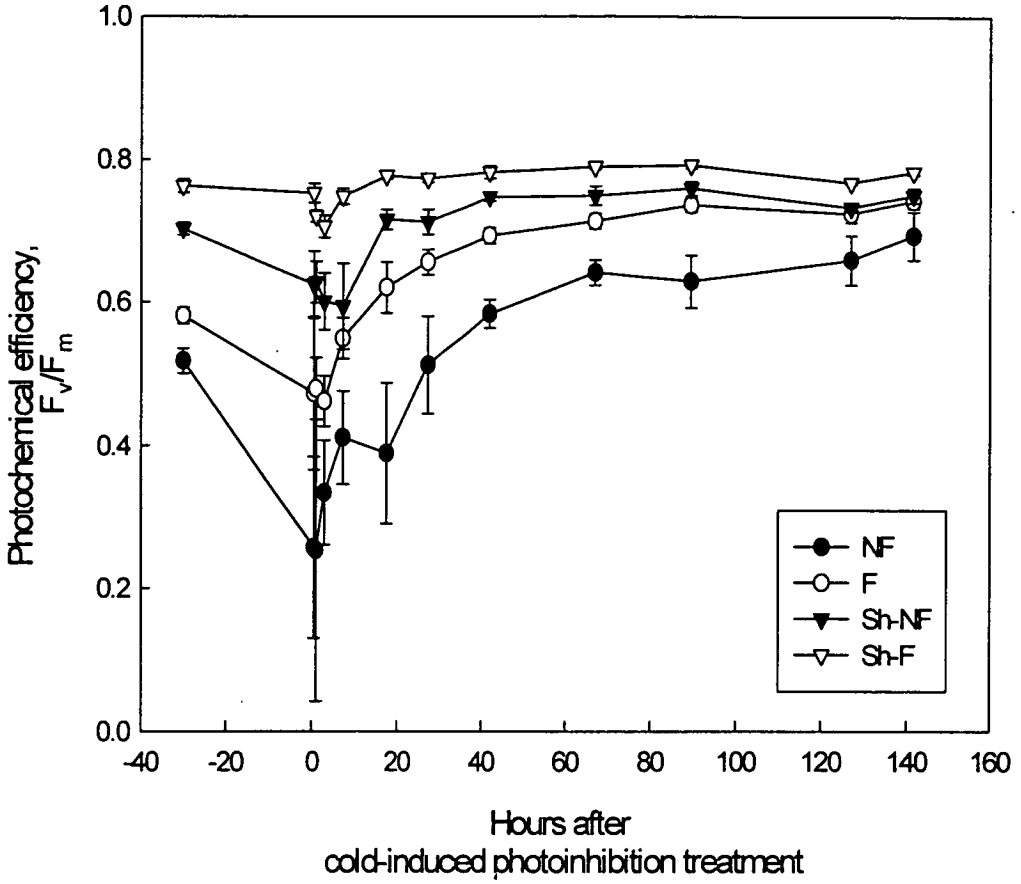


Figure 6.1. Changes in photochemical efficiency (F_v/F_m; dimensionless) with time after exposure of leaves of NF, F, Sh-NF and Sh-F *E. nitens* seedlings at time 0 to artificial warm, low light conditions. Three hours of photoinhibitory conditions were imposed before time 0. The first point on the graph represents levels after 24 h of dark adaptation at 21 °C. Bars indicate ± standard error.

Relaxation of xanthophyll cycle conversion state (A+Z/V+A+Z)

Immediately after cold-induced photoinhibition, xanthophyll conversion states (A+Z/V+A+Z) were highest in NF and F, lower in Sh-NF ($p < 0.005$) and lower again in Sh-F ($p < 0.05$) seedlings (Figure 6.2). Significant de-epoxidation of Z and A occurred in all treatments between 0 and 25 ($p < 0.005$) and 25 and 38 h ($p < 0.0001$) of the recovery period after cold-induced photoinhibition.

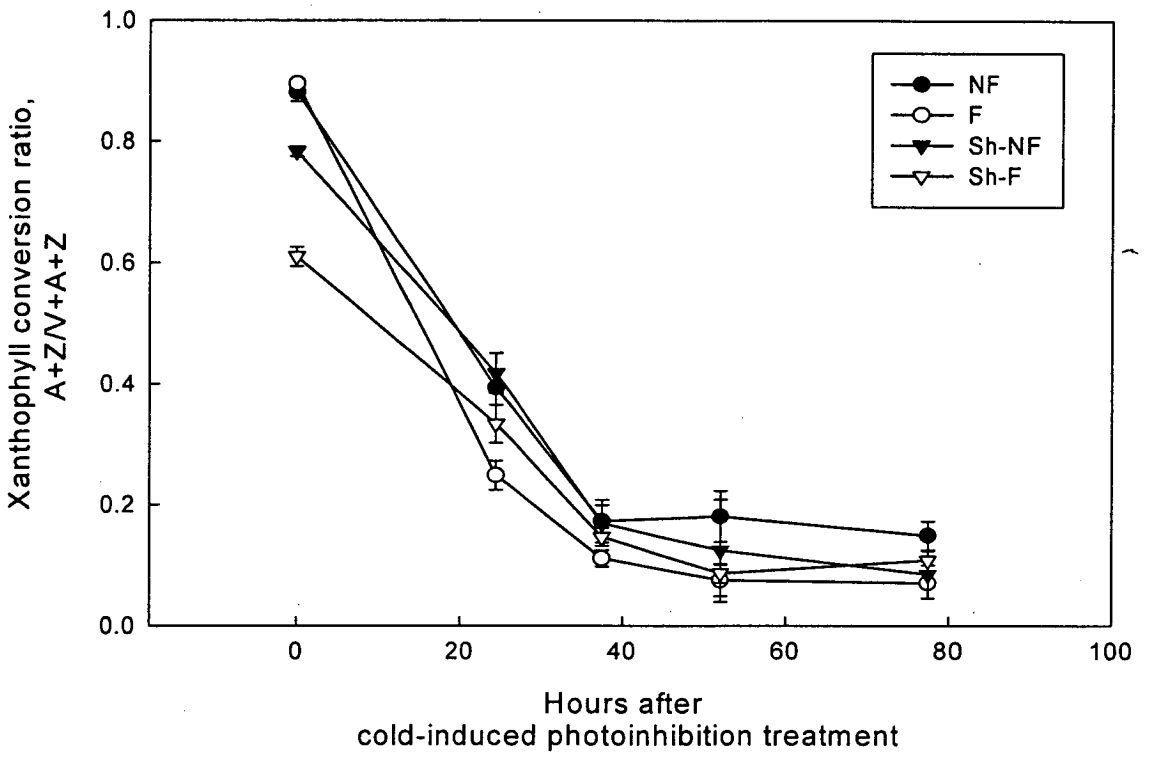


Figure 6.2. Changes in pre-dawn xanthophyll-cycle conversion state (A+Z/V+A+Z; dimensionless) after exposure of leaves from NF, F, Sh-NF and Sh-F *E. nitens* seedlings at time 0 to artificial warm, low light conditions. Bars indicate ± standard error.

Discussion

Major findings

This experiment has demonstrated that sustained nocturnal xanthophyll cycle engagement occurs in *E. nitens* seedlings during establishment in winter. The extent of this engagement was affected by nutrient status at planting (and thus foliar pigment levels) and the level of exposure to light after planting. After 24 h of exposure to artificial warm, low light conditions, sub-optimal F_v/F_m persisted in non-shaded NF and F seedlings. Photoinhibition induced by simulated cold morning conditions led to persistent depression of F_v/F_m and, concurrently, sustained increases in xanthophyll cycle conversion state (A+Z/V+A+Z) in all treatments. The ranking of the level of depression of F_v/F_m and the time of recovery to optimal levels was NF > F > Sh-NF > Sh-F. The ranking of A+Z/V+A+Z was NF = F > Sh-NF > Sh-F initially but there were no differences between treatments after 24 h.

Pigment compositions

Foliar pigment composition (other than the kinetics of A+Z/V+A+Z) was similar between treatments to that reported in Chapter 5 and are not discussed here.

Relaxation of F_v/F_m

Sustained depression of F_v/F_m was only slowly reversible under artificial conditions of low light and warm temperature. This has been similarly found in *Pseudotsuga menziesii* (Adams *et al.* 1994), *Pinus sylvestris* (Ottander *et al.* 1995) and *Eunonymus kiatschovicus* and *P. ponderosa* (Verhoeven *et al.* 1999). In the *E. nitens* treatments in this study the least photoinhibited seedlings took the shortest period to recover and vice versa. Correlation between recovery period and the degree of photoinhibition is

consistent with other reports involving comparisons of recovery period and severity of photoinhibition between leaves in winter and summer (Verhoeven *et al.* 1998; 1999) and leaves (Adams and Barker 1998) or cladodes (Barker *et al.* 1998) of different orientation.

The details of the mechanism resulting in slow recovery of F_v/F_m under warm conditions are unknown. Suggestions include conformational changes in D1 protein (Ohad *et al.* 1990) and phosphorylation of thylakoid proteins (Kim *et al.* 1997). A correlation between recovery of F_v/F_m and the synthesis of light harvesting complexes (LHC) and D1 protein in *P. sylvestris* has been reported (Ottander *et al.* 1995). It was suggested that synthesis of these proteins was indicative of a re-organization of the LHC. A 21% increase in ß-carotene content during recovery of F_v/F_m over 120 h in *P. ponderosa* has been proposed as consistent with the synthesis of LHC proteins (Verhoeven *et al.* 1999). No increase in ß-carotene was found in *E. nitens* during a recovery period of 142 h.

Relaxation of A+Z/V+A+Z

Sustained decreases in PSII efficiency have been associated with sustained increases of the xanthophyll cycle conversion state, A+Z/V+A+Z, in a range of evergreen species (Adams and Demmig-Adams 1994; 1995; Adams *et al.* 1995; Ottander *et al.* 1995; Jahns and Miehe 1996; Verhoeven *et al.* 1996; 1998; 1999; Adams and Barker 1998; Barker *et al.* 1998; Logan *et al.* 1998) as was found here with *E. nitens*. The strategy of retaining high A+Z/V+A+Z overnight enables immediate dissipation of excess light energy on mornings after frosts under clear skies which would otherwise have the potential to cause severe photodamage (Adams *et al.* 1994). Previous reports

have found that the severity of decrease in PSII efficiency is correlated to the increase in A+Z/V+A+Z. However, after 24 h from the initiation of recovery, treatments had similar A+Z/V+A+Z ratios in contrast to the respective F_v/F_m values which remained significantly different between treatments until 70 h of recovery. This lack of correlation, similarly observed in Chapter 5, may indicate that mechanisms other than the xanthophyll cycle account for decreased PSII efficiency.

Recovery of F_v/F_m over 72 h in foliage of *P. ponderosa* under artificial warm and low light conditions was associated with recovery of D1 protein and re-organisation of LHC from a high to low quenched state, indicating that these mechanisms may play a role in dissipation of excess light energy during periods of sustained xanthophyll activity (Ottander *et al.* 1995). The possible role of these mechanisms was discussed in detail in Chapter 5. Studies on leaves of *Oryza sativa* L. have similarly found a lack of correlation between A+Z/V+A+Z and F_v/F_m during recovery under warm, low light conditions (Xu *et al.* 1999) and it was concluded that there was no causal relationship between changes in F_v/F_m and A+Z/V+A+Z but that epoxidation of Z+A was related to photosynthetic electron transport activity. Recovery of electron transport activity depends on recovery of PS II (F_v/F_m) which indicates an indirect link between the two processes. Therefore during recovery under artificial warm, low light conditions the recovery processes of both F_v/F_m and A+Z/V+A+Z proceed independently during the slow phase once ATP-dependent reverse proton transport has (relatively rapidly) dissipated (Xu *et al.* 1999).

Conclusion

Sustained xanthophyll cycle engagement is employed as an overwintering strategy in *E. nitens* seedlings during establishment. Depression of F_v/F_m and its recovery is minimised and maximised, respectively, by high levels of foliar pigments which can be effected by nutrient loading before planting. Shading seedlings in the field further contributes to the same recovery kinetics. Xanthophyll cycle conversion accounts for some but not all decreases in PSII efficiency which indicates that other mechanisms may be quenching excess light energy. This was similarly concluded in Chapter 5.

Conditions which cause sustained engagement of the xanthophyll cycle can overtake its capacity to dissipate excess energy. Under such conditions, synthesis of increased levels of antioxidant compounds occurs. This is the subject of the following chapter.

Chapter 7. Physiological antioxidants and compounds deterring herbivory and in light attenuation?

Introduction

Photoinhibition, photodamage and antioxidants

Photodamage is caused by the production of reactive oxygen species if the absorption of light energy exceeds the plant's capacity to utilise or dissipate this energy (Wise and Naylor 1987). Scavenging antioxidant molecules (eg. carotenoids, tocopherols, ascorbate and glutathione) and enzyme systems (eg. super oxide dismutases [SODs]) neutralise these oxidizing species through electron donation and alleviate photodamage. Turnover rates of ascorbate and glutathione increase under conditions of increased nutrient limitation-induced photoinhibition (Polle *et al.* 1992; Logan *et al.* 1999) and increasing cold-induced photoinhibition (Polle *et al.* 1992; Polle and Rennenberg 1996). Anthocyanins have been hypothesised to act also as antioxidants (Yamasaki *et al.* 1996). In addition, they may have a role in the attenuation of light (Krol *et al.* 1995; Barker *et al.*1997; Woodall *et al.*1998).

Recent findings of imbalances between SODs and other enzymes providing photoprotection against reactive oxygen species prompted Cheeseman *et al.* (1999) to speculate that other antioxidant compounds were yet to be identified. Many polyphenolic compounds, such as flavonoids and tannins, which are known to have antioxidant properties, have been reported to occur in *Eucalyptus* leaves (Okamura *et al.* 1993a; 1993b; Cadahía *et al.* 1997; Conde *et al.*1997) and leaves of other species (Constantino *et al.* 1993; Lamaison *et al.*1993; Constantino *et al.* 1994; Veit *et al.* 1994; 1996; Crozier *et al.* 1997; Nawwar *et al.* 1997; Kofinas *et al.* 1998;

Sanchezmoreno *et al.* 1998; Gardner *et al.*1998; Grace *et al.*1998). The capacity of flavonoids to act as antioxidants has been shown to depend on the number of hydroxyl groups (Hodnick *et al.* 1988) and their ability to scavenge free radicals is related to the relative degree of conjugation (Ariga and Hamano 1990). This capacity has been of interest in human nutrition (Constantino *et al.* 1994; Nakayama 1994; Miller and Rice-Evans 1997; Gardner *et al.* 1998; Sanchezmoreno *et al.*1998) but their potential physiological role in plants has received only scant attention (Rice-Evans *et al.*1996). Galloylglucoses, part of the tannin family, have demonstrated antioxidant properties (Okamura *et al.* 1993b; Hagerman *et al.* 1998), numerous hydroxyl groups and a greater capacity than flavonoids as antioxidants (Hagerman *et al.* 1998; Gardner *et al.* 1999) due to a greater amount of conjugation (Hagerman *et al.* 1998). It has similarly been demonstrated that chlorogenic acid (CGA) is a superior antioxidant compared to the closely related caffeic acid: CGA acts as a powerful antioxidant which minimises photodamage during periods of severe cold-induced photoinhibition in *Mahonia repens* (Grace *et al.* 1998). High levels of galloylglucose were reported in leaf extracts of *Eucalyptus rostrata*: the antioxidant activity of *E. rostrata* extracts was high relative to that found in 15 other *Eucalyptus* species tested (Okamura *et al.* 1993b).

Ecological implications of tannins

Previous analyses of tannins in the investigation of the effects of foliar polyphenolic compounds on feeding choices of herbivores (Larsson *et al.* 1986; Basey and Jenkins 1993; Hartley *et al.*1995; Hartley *et al.* 1997; Cork and Catling 1996) have yielded inconclusive results. A primary reason for this is that methods used for their identification and quantification have not been able to discriminate between different

polyphenolic families. A recent investigation using a more discriminatory method has identified diformylphloroglucinols (DFPs) (Pass *et al.*1998) as the family of polyphenolics in *Eucalyptus* foliage that deters feeding of the common ringtail possum (*Pseudocheirus peregrinus*) and koala (*Phascolarctos cinereus*) (Lawler *et al.* 1998). Sideroxylonals have been identified as a sub-group of the DFPs that confer deterrence (Eschler and Foley 1999).

Identification and analysis of tannins

High pressure liquid chromatography (HPLC) interfaced to electrospray ionization mass spectrometry (ESI-MS) with negative ion detection has been used in the identification of medicinal compounds in plants (Nawwar *et al.* 1997) and of polyphenolic compounds which may act as protective agents against microbial or animal predators (Wolfender *et al.*1994; Barry *et al.*in press; Davies and Barry in press). HPLC/ESI-MS allows also accurate identification and analysis of specific galloylgluocose and sideroxylonal compounds and quantitative studies of their variation in response to growing environment, such as incident light levels and herbivory, and plant physiological condition, such as foliar nutrient content and photoinhibition (Close *et al.* 1999; in press). Changes in levels of galloylglucoses, sideroxylonals and flavonoids in relation to these variables have not previously been reported.

Hypotheses tested

This paper identifies possible mechanisms that eucalypt seedlings employ to reduce physiological and physical damage, thereby maximising their potential for survival and growth. In so doing, the use of HPLC/ESI-MS is demonstrated as a powerful tool

for the identification and analysis of the foliar flavonoids, galloylglucoses and sideroxylonals. Three hypotheses were postulated and then tested;

- that polyphenolic compounds having potential as light attenuators and antioxidants are present in foliar extracts of eucalypt seedling leaves;
- that temporal variation of anthocyanins correlates with severity of cold-induced photoinhibition and;
- that levels of galloylglucoses and flavonoids correlate with physiological measures of foliar nutrition, incident light and cold-induced photoinhibition.

Sideroxylonals, extracted and analysed by methods used for galloylglucoses, were investigated following an herbivory event during the course of the experiment.

Materials and methods

Experimental site, plant material, treatments and sampling methods

The experimental site, plant material, treatments and sampling methods for the various analyses were detailed in Chapter 4.

Galloylglucose, flavonoid, sideroxylonal and anthocyanin analyses

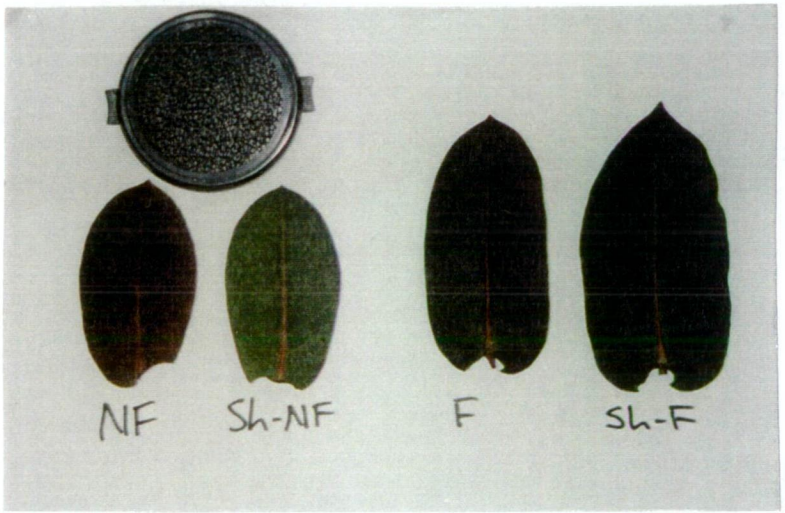
Five leaf discs from each of the most recently expanded leaf pair of three randomly selected seedlings of each treatment were sampled on 21 May (pre-planting), 23 June, 8 October 1998 and 20 January 1999 (2, 15 and 29 weeks after planting respectively). The extractions and chromatographic and spectrometric analyses involving HPLC/UV, HPLC/ESI-MS and MS/MS were described in Chapter 2. Methods used for the identification and semi-quantification of temporal changes in galloylglucoses,

flavonoids, sideroxylonals and anthocyanins were also detailed in Chapter 2. Leaves typical of those assessed on 8 October are shown in Plate 7.1.

Visible/near infra red spectroscopy (VIS-NIRS)

Procedures used for VIS-NIRS analysis were detailed in Chapter 2. On 8 October 1998, the two most recently expanded leaves of 10 randomly selected seedlings of each treatment were sampled for VIS-NIRS analysis. Leaves typical of those assessed are shown in Plate 7.1.

Plate 7.1. Leaves typical of those analysed for galloylglucose, flavonoid, sideroxylonal, anthocyanin and VIS-NIRS on 8 October 1998.



Nitrogen analysis

Leaves were analysed for determination of foliar N concentration as detailed in Chapter 2. Samples were collected on 2 June, 21 September 1998 and 25 January 1999 (Table 7.1).

Table 7.1. Total foliar nitrogen content (% DW) of *E. nitens* seedling foliage during establishment.

	Pre-Planting	15 Weeks	29 Weeks
NF	1.17 ± 0.05	1.00 ± 0.03	2.27 ± 0.09
F	2.13 ± 0.04	1.43 ± 0.08	2.06 ± 0.04
Sh-NF	1.17 ± 0.05	0.87 ± 0.05	2.28 ± 0.16
Sh-F	2.13 ± 0.04	1.48 ± 0.03	1.94 ± 0.07

Gas exchange

Procedures used for gas exchange were detailed in Chapter 2. Gas exchange results are those previously described in chapter 4.

Statistical analysis

Differences in A_{max}, foliar sideroxylonal, galloylglucose, flavonoid, anthocyanin, and N levels were analysed as detailed in Chapter 2.

Results and discussion

HPLC/UV and HPLC/ESI-MS analysis

A typical chromatogram for foliar tannin analysis indicated a high level of complexity (Figure 7.1). Foliar tannin extracts from other species are also complex (Okamura *et al.* 1993a; Wolfender *et al.* 1994) and similar chromatograms have been reported for foliar extracts from *Eucalyptus camaldulensis*, *Eucalyptus globulus* and *Eucalyptus rudis* (Conde *et al.* 1997; Cadahía *et al.* 1997). Retention times and investigation of UV spectra detected via photodiode array indicated two families of compounds,

galloylglucoses and sideroxylonals (Figure 7.1). Each family was chromatographically distinct. These tannins were not identified in previous investigations which utilised HPLC/UV detection methods only (Conde *et al.* 1997; Cadahía *et al.* 1997). MS total ion current (TIC) chromatograms from the HPLC/ESI-MS analysis of the same extracts (Figure 7.2) revealed a similar pattern of peaks to that of tannins detected by UV at 280 nm. Examination of MS data indicated that co-elution of several tannins and flavonoids was occurring, making it impossible to interpret the results by UV detection alone.

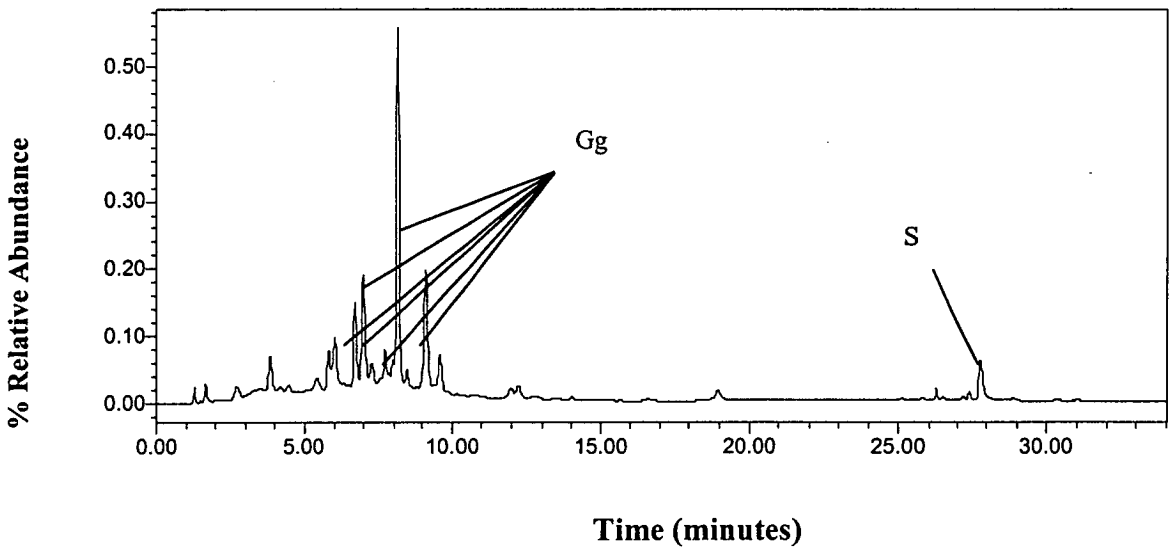


Figure 7.1. HPLC/UV chromatogram of acidified methanol extracts measured at 280 nm indicating a range of galloylglucoses (Gg) and sideroxylonal A (S).

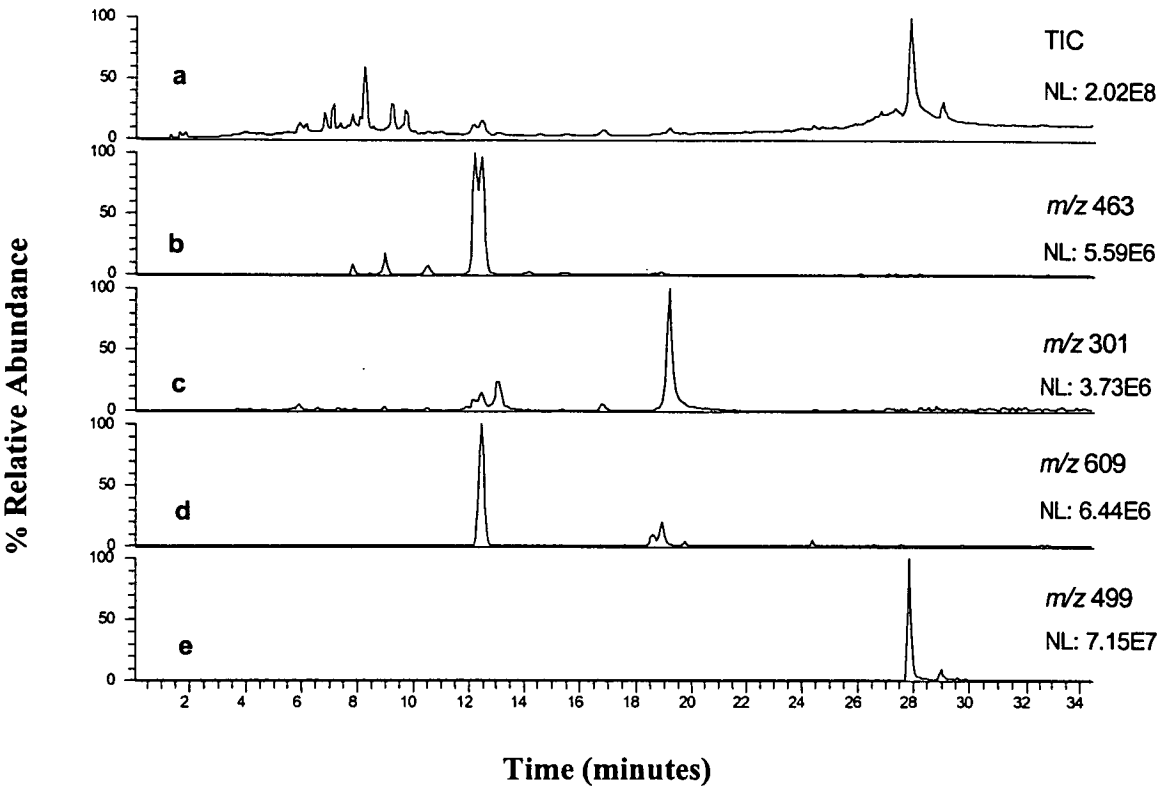


Figure 7.2. Negative ion electrospray total ion current (TIC) chromatogram (a), quercitin glycosides at *m/z* 463 (b), quercitin at *m/z* 301 (c), rutin at *m/z* 609 (d), and sideroxylonal A and B at *m/z* 499 (e) indicating co-elution of flavonoid and sideroxylonal peaks. Normalised level (NL) indicates the absolute measure of the abundance scale.

The application of ESI-MS to the study of polyphenolic compounds has been demonstrated previously (Nawwar *et al.* 1997; Barry *et al.* in press; Davies and Barry in press). Negative ions yield abundant signals conferring very low detection limits. Comparison of retention times and major ions obtained using HPLC/ESI-MS with authentic standards allowed identification of sideroxylonal A and B and penta-

galloylglucose (Figure 7.3a, e). Other major peaks (Figure 7.3b, c, d) were assigned as various isomers of di-, tri-, and tetra-galloylglucose. The degree of substitution of the galloylglucoses was indicated by molecular weights obtained by MS. Confirmation was obtained through fragmentation via collision-induced disassociation (CID) yielding MS/MS daughter ions produced by losses of 152 (gallic acid – H_2O) and 170 (gallic acid) (Davies and Barry in press). Compounds identified in this way comprised ≈ 75 % of total UV absorption detected in extracts at 280 nm. The identification of penta-galloylglucose and assignment of di, tri, and tetra-galloylglucoses accords with the identification of 1, 2, 6-tri-o-galloyl-β-D-glucose in foliar extracts of *E. rostrata* using NMR techniques (Okamura *et al.* 1993a, b). The positive identification of sideroxylonal A and B in this study accords with previous identification of sideroxylonal A and B in *E. nitens* foliar leaf extracts (Eschler and Foley 1999). The accuracy of identification of compounds using ESI-MS molecular weights obtained with MS and CID MS/MS data has been demonstrated during assay of complex foliar extracts which validated identifications using NMR techniques (Nawwar *et al.* 1997).

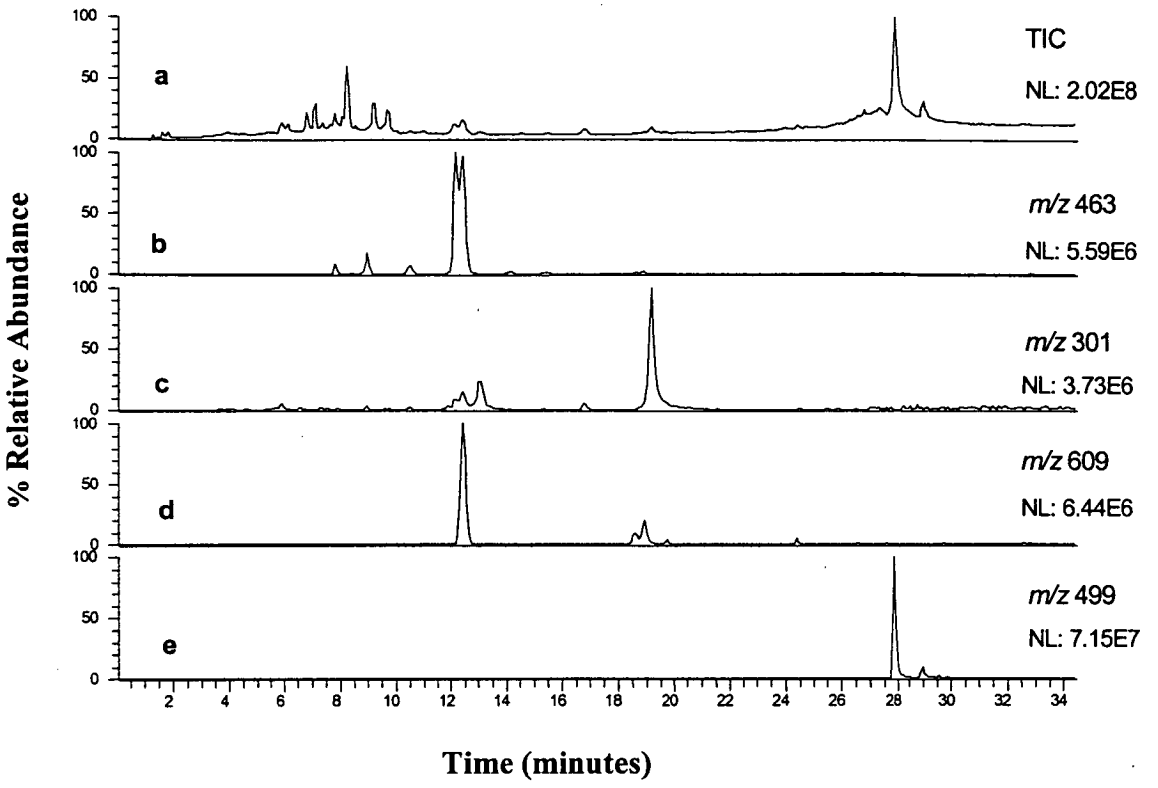


Figure 7.3. Negative ion electrospray chromatograms of TIC (a) indicating a range of galloylglucoses (Gg) and sideroxylonal A and B (S), $[M-H]^-$ ions for di-galloylglucoses at *m/z* 483 (b), tri-galloylglucoses at *m/z* 635 (c), tetra-galloylglucoses at *m/z* 787 (d), and penta-galloylglucose at *m/z* 939 (e). Normalised level (NL) indicates the absolute measure of the abundance scale.

A typical UV chromatogram at 370 nm for flavonoid analysis (Figure 7.4) revealed far less complexity than that for the galloylglucoses and sideroxylonals. The chromatogram had three major peaks similar to that reported for *E. camaldulensis*, *E. globulus* and *E. rudis* (Cadahía *et al.* 1997), quercitin, rutin and a second quercitin-glycoside. The unidentified major quercitin glycoside constituent and other minor

peaks indicating quercitin-glycosides were identified by characteristic $[M-H]^-$ ions at *m/z* 463 and by MS/MS spectra (Figure 7.5). The 3 major peaks accounted for ≈ 85% of UV absorption at 370 nm. A range of flavonol glycosides have been previously isolated from foliar pigment extracts of *E. rostrata* using NMR techniques (see Okamura *et al.* 1993a, b).

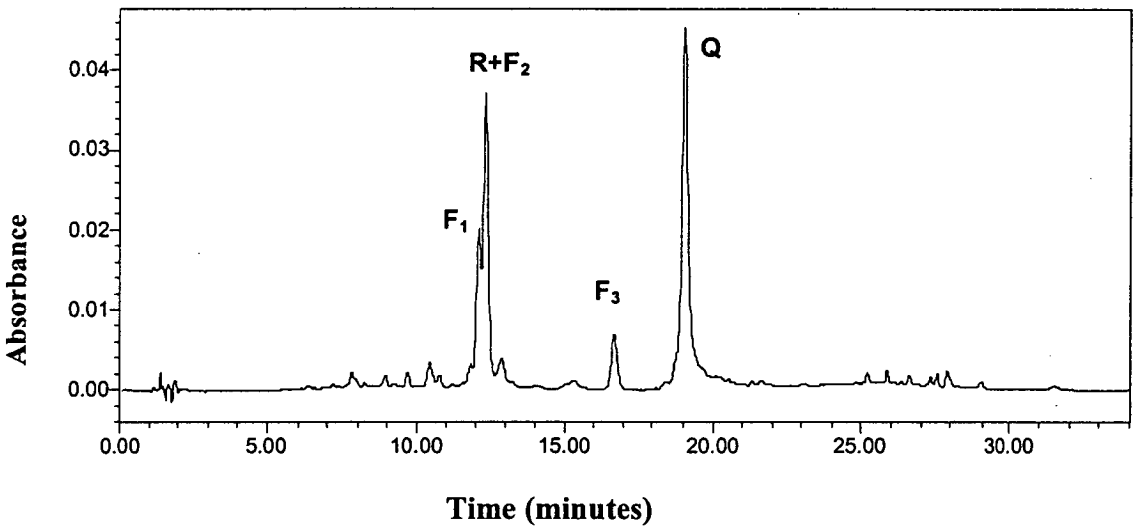


Figure 7.4. HPLC/UV chromatogram of acidified methanol extracts measured at 370 nm indicating 3 major peaks of rutin and 2 unknown quercitin-glycosides (R, F_1, F_2 respectively), an unknown flavonol glycoside (F_3), quercitin (Q) and minor peaks of unknown flavonol-glycosides.

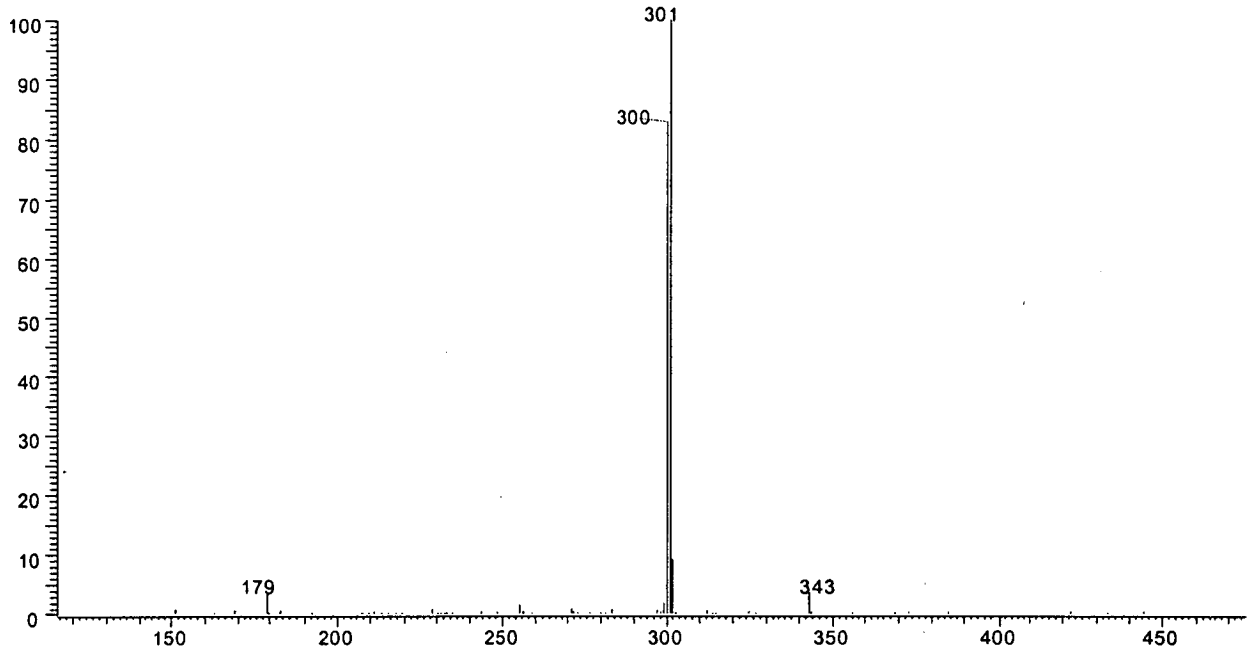


Figure 7.5. Mass spectrum of CID MS/MS daughter ions produced by losses of glycoside residue from unidentified flavonol-glycosides at $[M-H]^-$ 463.

Temporal variation of anthocyanins

The anthocyanins, cyanidin-3-glycoside and cyanidin-3,5-diglycoside, were similar between treatments except at 15 weeks after planting (Figure 7.6a). At this time total anthocyanins were significantly higher ($p < 0.005$) in NF compared to other treatments. The pattern of change of anthocyanin level was not related to that of galloylglucose or flavonoids (Figures 7.6c, d). This is consistent with anthocyanins not acting primarily as antioxidants in foliage, as has been suggested previously (Yamasaki *et al.* 1997). Antioxidant activity was reported in *Mahonia repens* L. foliage both in the presence and absence of anthocyanins (Grace *et al.* 1998). VIS-

NIRS of leaves, sampled 15 weeks after planting, indicated that NF seedlings absorbed significantly higher levels of radiation between 400 and 590 nm relative to other treatments (Figure 7.7). This was related to significantly higher levels of anthocyanin relative to other treatments (Figure 7.6a) during the period of greatest severity of cold-induced photoinhibition (see Figure 5.3). Higher levels of anthocyanin and absorption of radiation in foliage of the NF treatment indicate that anthocyanins may play a physiological role of light attenuation in *E. nitens*. Other recent investigations have suggested such a role (Krol *et al.* 1995; Barker *et al.* 1997; Pietrini and Massacci 1998; Woodall *et al.* 1998).

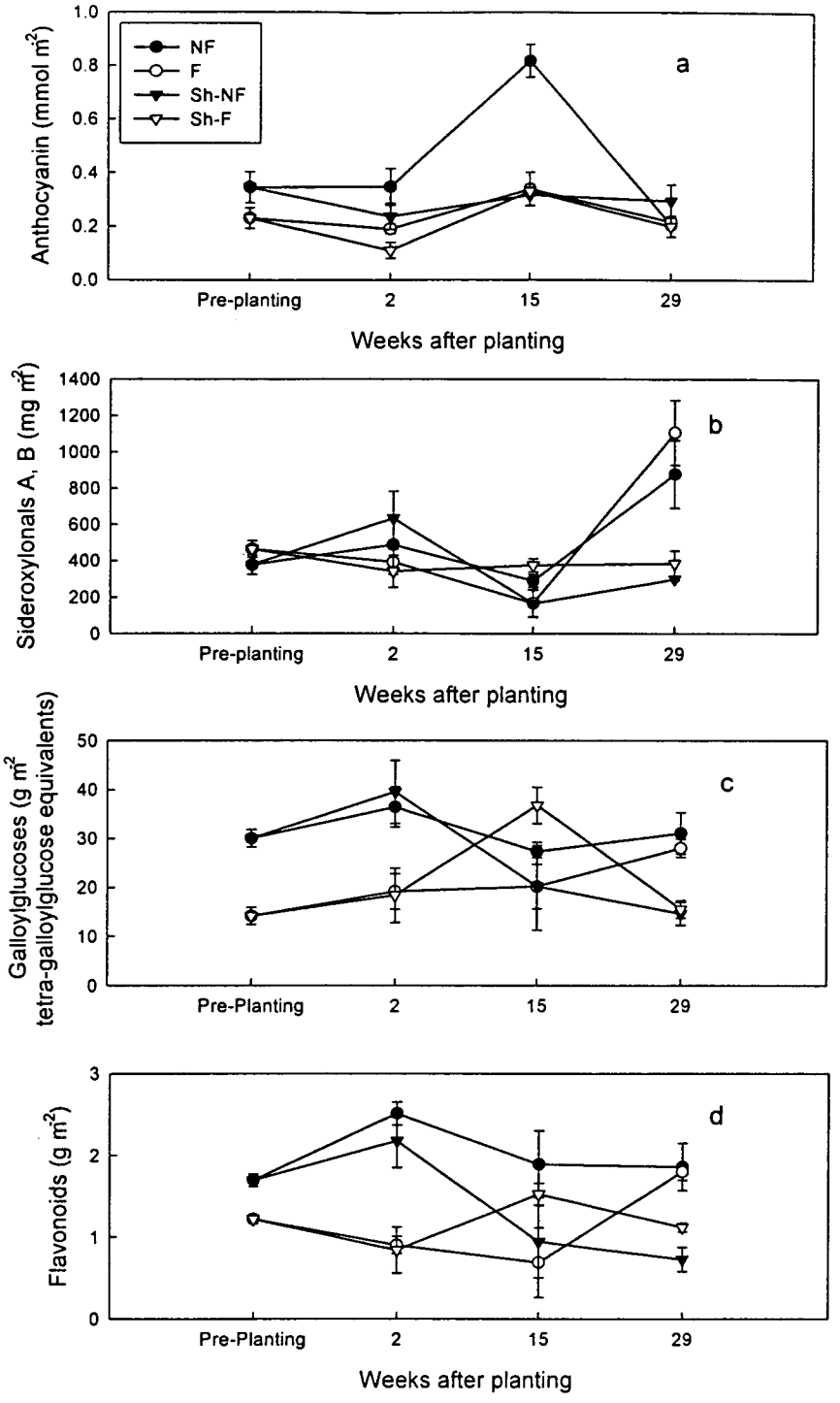


Figure 7.6. Total anthocyanins (mmol m^{-2}) (a), sideroxylonal A and B (ng m^{-2}) (b), total galloylglucoses (tetra-galloylglucose equivalents mg m^{-2}) (c), and total flavonoids (ng m^{-2}) (d) measured on 21 May (pre-planting), 23 June (2 weeks after planting), 8 October 1998 (15 weeks after planting), and 20 January 1999 (29 weeks after planting) of *E. nitens* foliar extracts.

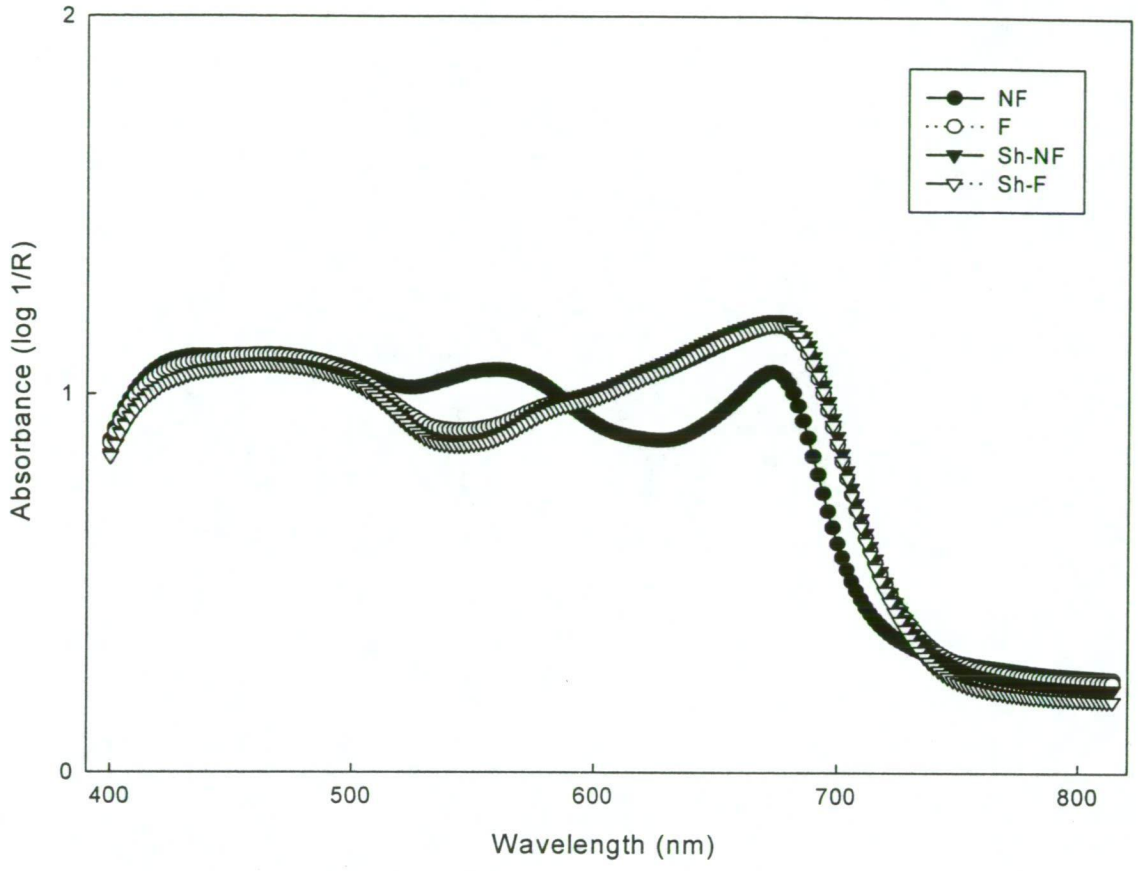


Figure 7.7. Near infrared spectra (VIS-NIRS) of leaves of *E. nitens* seedlings sampled 15 weeks after planting.

Temporal variation of sideroxylonals

Sideroxylonal showed little temporal variation before planting and at weeks 2 and 15 after planting, but varied between treatments 29 weeks after planting (late January) (Figure 7.6b). At this time both NF and F leaf extracts had significantly higher levels of sideroxylonal than those recorded earlier ($p < 0.04$, $p < 0.01$ respectively) and compared to the shaded treatments ($p < 0.04$, $p < 0.02$ respectively). Invertebrate browsing [(Coleoptera; Chrysomedidae) *Chrysophtherta bimaculata* (Olivier)] was observed at this time on the exposed seedlings but did not occur in Sh-F and Sh-NF

treatments due to protection from the shadecloth tree shelters. Current evidence indicates that high sideroxylonal levels in unbrowsed *Eucalyptus* foliage deters vertebrate browsing (Lawler *et al.* 1998). Our results suggest that increased sideroxylonal levels could be an induced response in *E. nitens* foliage to invertebrate browsing. Alternatively, these increased levels may be associated with increased light absorption at week 29 compared to week 15 and the correlation in response to browsing by *C. bimaculata* purely co-incidental.

Temporal variation of galloylglucoses and flavonoids

There were large and similar patterns of change with time in levels of galloylglucoses and flavonoids (Figures 7.6c, d). Galloylglucoses were approximately 150% and 100% higher in NF ($p < 0.002$) and Sh-NF ($p < 0.01$) compared to F and Sh-F treatments before planting and 2 weeks after planting, respectively (Figure 7.6c). Similarly, flavonoids were 25% and up to 300% higher in NF ($p < 0.04$) and Sh-NF ($p < 0.001$, $p < 0.04$ respectively) compared to F and Sh-F treatments before planting and 2 weeks after planting, respectively (Figure 7.6d). Differences in galloylglucose and flavonoid levels at this time were probably induced by fertiliser regime in the nursery and related to differences in foliar nitrogen concentrations (Table 7.1). There was large variability between samples and no well defined pattern between treatments taken at 15 weeks after planting. By 29 weeks after planting non-shaded treatments (NF, F) had galloylglucose levels approximately 100% higher ($p < 0.03$, $p < 0.01$ respectively) than the shaded (Sh-NF, Sh-F) treatments (Figure 7.6c). The same trend of non-shaded compared to shaded treatments was apparent in flavonoid levels ($p < 0.005$) (Figure 7.6d). This indicates that the effects of fertiliser level had been replaced by the effects of level of incident light.

High levels of galloylglucoses and flavonoids prior to, and just after, planting were associated with low maximum net photosynthetic assimilation rates, A_{max}, for NF treatments and vice-versa for F treatments (see Figure 5.3). Thus nutrient starvation in the nursery induced low foliar N levels and decreased A_{max} in NF compared to F treatments. Foliar N concentration and A_{max} decreased for several weeks in both NF and F treatments. This was followed by a period of nutrient acquisition which removed differences in foliar nutrient levels between treatments by 29 weeks after planting (Table 7.1). At this time A_{max} was high and there were no significant differences between treatments (Figure 7.7) but incident light levels differed by 50% between shaded and non-shaded treatments. It appears that there is a relationship between high foliar galloylglucose and flavonoid levels and attainment of similar A_{max} in non-shaded compared to shaded treatments. These results concur with a study that investigated changes in CGA (a polyphenolic antioxidant) levels between shaded and non-shaded plants of *M. repens* both in winter and summer (Grace *et al.* 1998). This comparison revealed an approximately two-fold increase of CGA between summer and winter: in *E. nitens* there was a two-fold difference in galloylglucoses and flavonoids between NF and F treatments (Figures 7.6c, d). In both comparisons, the higher level of polyphenols was associated with the induction of increased levels of photoinhibition (see Figure 5.3). Likewise, in summer, comparison of shaded and non-shaded *M. repens* yielded a two-fold difference in CGA level, the same finding as for galloylglucose and flavonoid levels in shaded and non-shaded *E. nitens* foliage. The similarity of change in flavonoid and galloylglucose levels is notable given the 4-fold difference in absolute levels between them. The synthesis of greater

galloylglucoses than flavonoids may be related to the superior antioxidant capacity of the galloylglucoses.

No investigations of changing galloylglucose or flavonoid levels associated with photoinhibition have been reported to date. Many studies on UV radiation report increased levels of UV absorbing flavonoids associated with depressed photosynthesis (Reuber *et al.*1996; Ambasht and Agrawal 1997; Moorthy and Kathiresan 1997; Ambasht *et al.* 1998; Sharma *et al.* 1998). However the converse, increased photosynthesis, has been reported to result from exclusion of UV which decreased flavonoid levels (Lingakumer *et al.* 1999). In *E. nitens* both NF and F, and Sh-NF and Sh-F treatments were exposed to identical UV levels. Thus UV radiation cannot explain the observed differences in galloylglucose and flavonoid levels before planting and 2 weeks after planting. However, the shadecloth may have decreased UV relative to non-shaded treatments and contributed to differences in these polyphenolic compounds 29 weeks after planting.

The ecological and physiological roles of galloylglucoses and sideroxylonal

The growth rate hypothesis (Coley *et al.* 1985) and the carbon:nutrient balance (CNB) hypothesis (Bryant *et al.* 1983; Bryant *et al.* 1987; 1991) argue that broad tannin levels provide a 'quantitative' defense against herbivory, which depends on the ecological niche and conditions limiting growth. The CNB hypothesis predicts that when nutrient levels are limiting, the products of carbon fixation accumulate relative to the levels of nutrients available for plant growth and compounds used for defence against herbivory, such as polyphenolics, increase. Under light limiting conditions, the lower availability of fixed carbon is predicted to lead to lower levels of

polyphenolics. This work has shown that under conditions of light and nutrient limitation, *E. nitens* seedlings experience lower and higher levels of photoinhibition, respectively. There are many investigations testing the CNB hypothesis that question its validity (eg. Waterman *et al.* 1984; Hemming and Lindroth 1999). The shortcomings of the tannin-herbivory defence hypotheses have been highlighted by identification of sideroxylonals (Eschler and Foley 1999), which are the chemical sub-group of DFPs proposed to be responsible for deterrence against herbivory in eucalypt foliage (Lawler *et al.* 1998; Pass *et al.* 1998) but represent only a fraction of total phenolics in eucalypt leaves. In this study, of the ≈ 70% of tannins identified by HPLC/ESI-MS and MS/MS data, sideroxylonals represented ≈ 1% of total polyphenolics absorbing at 280 nm while galloylglucoses comprised the remaining ≈ 99%.

Conclusion

Studies of compounds that confer deterrence to herbivory have reported changes in levels of polyphenolic antioxidants which are metabolised in response to photoinhibition. Total levels of polyphenolic compounds in leaves change also in response to small changes in the level of incident light (Newbery and de Foresta 1985; Mole *et al.* 1988). Given the finding here that total levels of polyphenols constitute ≈ 99% galloylglucoses and ≈ 1% sideroxylonals in *E. nitens* foliage, it is suggested that tannins do not act solely as a quantitative defence against herbivory but are the summation of antioxidant activity and compounds which deter herbivory.

In this chapter it was observed that foliar anthocyanin synthesis was related to severity of cold-induced photoinhibition and not to the level of antioxidant activity. Near infra

red spectroscopy (NIR) data indicated that leaves of high anthocyanin content may attenuate light absorbed by the leaf. However, physiological differences other than that of anthocyanin levels existed between treatments. For this reason the physiological role of foliar anthocyanin was investigated under controlled conditions which rapidly induced anthocyanin synthesis. This experiment is the subject of the following chapter.

Chapter 8. Effects of rapid induction of cold-induced photoinhibition

Introduction

Physiology of anthocyanins

The ecophysiological role of foliar anthocyanins is a contentious issue. Leaf colour has been described as a by-product of the general metabolism of flavonoids, the colour being expressed by anthocyanins (Lee *et al.* 1987). Anthocyanin has been described as an antioxidant (Rice-Evans *et al.* 1996; Yamasaki *et al.* 1996; Wang *et al.* 1997); as a UV protectant (Lee and Lowry 1980; Burger and Edwards 1996; Jayakumar 1999); and as providing protection from visible light through light attenuation (Gould *et al.* 1995; Krol *et al.* 1995; Barker *et al.* 1997; Dodd *et al.* 1998; Pietrini and Massacci 1998; Mendez *et al.* 1999; Neill and Gould 1999). Evidence for UV protection may in fact be evidence of visible light protection as UV-A and UV-B have recently been shown to significantly contribute to photoinhibition (Krause *et al.* 1999). The role of light attenuation has attracted the most support from a wide range of sources. Individuals of *Begonia pavonina* Ridl. and *Triolena hirsuta* Triana, understorey natives from Malaysia and Costa Rica respectively and containing either high or low levels of foliar anthocyanin, have been compared. F_v/F_m and net photosynthesis were found to be higher in red compared to green individuals (Gould *et al.* 1995). Tolerance to photoinhibition of *Pinus banksiana* Lamb. seedlings was correlated with the accumulation of anthocyanin (Krol *et al.* 1995). Reflectance of light, conferring photoprotection, was correlated to the anthocyanin content of juvenile foliage of *Cotyledon arbiculata* (Barker *et al.* 1997) and during leaf development of *Syzgium luehmannii* and *S. wilsonii* (Woodall *et al.* 1998). The most conclusive demonstration of a link between anthocyanins and the attenuation of light

has been with *Zea mays* L., a species in which anthocyanin content progressively increases with decreasing growth temperature under constant conditions of high light. Anthocyanin absorption spectra were independently quantified and shown to linearly correlate with leaf absorptance between 400 and 600 nm. This demonstrated that the concentration of anthocyanins can vary with severity of excess light, moderating the light available to the chloroplasts (Pietrini and Massacci 1998). The effects of anthocyanin on light absorption were consistent across six unrelated New Zealand species that bore red leaves (Neill and Gould 1999).

Anthocyanin in seedlings

Foliar anthocyanin production in young conifer seedlings has been reported to result from exposure to low temperature and/or nutrient deprivation (Nozzolilo *et al.* 1989; Toivonen *et al.* 1991; Krol *et al.* 1994). It has long been observed in young eucalypt foliage (Sharma and Crowden 1974) and occurs in foliage of *E. nitens* and *E. globulus* seedlings in response to both low temperatures and/or nutrient deprivation (Close *et al.* 1999; 2000). Both these factors are associated with high levels of photoinhibition.

Anthocyanin and cold-induced photoinhibition

The association of foliar anthocyanin production with cold-induced photoinhibition has been a recurring theme in previous experiments. In Chapter 3 the dynamics of synthesis and de-synthesis of foliar anthocyanins was shown to be related to events causing cold-induced photoinhibition and foliage high in anthocyanin was observed to resist photodamage of leaf tissues. In Chapter 5, it was demonstrated that xanthophyll cycle activity was related to photoprotection during relatively mild, but not severe, photoinhibitory conditions when foliar anthocyanin levels increased dramatically.

This implied an association between anthocyanin and photoprotection which is not accounted for by xanthophyll cycle activity. The non-fertilised treatments, which had the greatest levels of foliar anthocyanin, were associated with higher levels of sustained xanthophyll activity in Chapter 6. In Chapter 7, the production of antioxidants was not related, in these treatments, to anthocyanin production which increased later and was associated with increased attenuation of visible wavelengths of light. However, the non-fertilised seedlings were different morphologically, limiting the confidence with which light attenuation could be attributed directly to anthocyanin production.

Hypotheses tested

An experiment was designed under controlled-growth conditions known to rapidly induce anthocyanin synthesis. Five hypotheses were postulated and then tested in this experiment;

- that the induced cold-induced photoinhibition, as measured by F_v/F_m, NPQ and xanthophyll cycle conversion states will be in the order of magnitude Sh-NF > NF > Sh-F > F;
- that increased cold-induced photoinhibition would induce increased levels of galloylglucoses and flavonoids but not sideroxylonals;
- that shaded would synthesise more anthocyanin than non-shaded treatments in response to increased cold-induced photoinhibition;
- that during the 14 days in the growth chamber at 8 °C minimal changes would occur in pigments other than anthocyanins in treatments not already high in foliar anthocyanin and;

- that different ETR response curves and VIS-NIRS spectra, before and after growth chamber treatment, would indicate attenuation of light mediated by anthocyanins.

Materials and methods

Plant material

Seedlings were raised from N2203 seedlot (North Forest Products Pty. Ltd. improved seed) in 85 cm^3 plugs in the North Forest Products' Somerset nursery. Seed was germinated on 27 September, 1998. Seedlings received Aquasol®every 10 days (solution concentration 1100-1500 mS). On 15 June 1999, seedlings were moved to a second nursery in Hobart. Half the seedlings were fed twice weekly with Aquasol® (concentration as above; referred to as the fertilised [F] treatment) while the others were starved of nutrients (referred to as the non fertilised [NF] treatment). Half the NF and F seedlings were raised under 50 % shadecloth whilst the other half were exposed to ambient conditions until 6 August after which they were transferred to a Thermoline (Sydney, Australia) growth chamber fitted with 400 W GE Koloarc MBID 400/T/H tubular metal halide lamps (Budapest, Hungary).

Experimental treatments

Daytime irradiance and leaf temperatures (measured with the PAM-2000) were 1460 μmol m^{-2} s^{-1} and 21.0 °C (non-shaded) and 470 μmol m^{-2} s^{-1} and 18.4 °C (shaded) in the nursery and 580 μmol m^{-2} s^{-1} and 14.4 °C in the growth chamber (Table 8.1). Growth chamber conditions were pre-programmed to 16 and 8 h light and dark, respectively. Spectral quality and quantity of the non-filtered halide lamps are detailed in Appendix 7. It was observed previously that the light conditions in the

growth chamber rapidly induced foliar anthocyanin synthesis in *Eucalyptus regnans* seedlings (T. Brodribb pers. comm.).

Forty seedlings of each treatment were arranged in a completely randomised design within the growth chamber. The shorter, non-fertilised seedlings were placed on wooden blocks and the less vertical stems and leaves of fertilised seedlings were supported to ensure uniform conditions of incident light on leaves of all treatments.

Seedlings remained in the growth chamber from days 0 – 12. From days 13 - 17 seedlings were exposed to warm (~ 21.0 °C), low light (~ 10 μmol m^{-2} s^{-1}) conditions.

Table 8.1. Irradiance and temperature conditions of seedlings before and during experimentation.

	Nursery in Hobart	Growth chamber
Irradiance (μmol m^{-2} s^{-1})		
Non-shaded	1460	580
Shaded	470	580
Leaf day temperature (°C)		
Non-shaded	21.0	14.4
Shaded	18.4	14.4
Leaf night temperature (°C)	9.8	8.0

Chlorophyll fluorescence, near infra-red spectroscopy and pigment analysis

F_v/F_m, during the final hour of the dark period, and NPQ, during the final hour of the light period, were measured (as detailed in Chapter 2) within the growth chamber on

three seedlings randomly selected from each treatment. Immediately after measurement of F_v/F_m, four leaf discs from each of the most recently expanded leaf pairs of three randomly selected seedlings (but not those used for F_v/F_m and NPQ measurements) of each treatment were sampled. Leaf discs were sampled pre-dawn before seedlings were arranged in the growth chamber (day 0), during the final hour of darkness in the growth chamber on days 2, 4, 8, and 12, and after 4 days under conditions of low light and warm temperature on day 16 for pigment analysis. The two most recently expanded leaves of 10 randomly selected seedlings of each treatment were excised on days 0 and 17 after GCT started, immediately placed in covered petri dishes containing moistened paper towelling, and stored at 4 ± 1 °C prior to VIS-NIRS analysis. Procedures used for pigment and VIS-NIRS analyses were detailed in Chapter 2.

Pigment extraction and analysis

Two of the four discs sampled were selected at random and extracted for chlorophyll, carotenoid, galloylglucose, sideroxylonal and anthocyanin determination (as detailed in Chapter 2). Due to the relatively low pigment concentration in these extractions, 10 µL of sample was injected into the HPLC.

Electron transport rate (ETR) response curves

Leaves were illuminated with white (actinic) light for 10 min at approximately 100 µmol m^{-2} s^{-1} to ensure phototsynthesis was fully induced. Actinic light intensity was then lowered to approximately 10 µmol m^{-2} s^{-1} and increased stepwise to approximately 950 µmol m^{-2} s^{-1} after 10 minutes at each light intensity. During the last minute of illumination at each light intensity, quenching parameters were assessed

with a saturation pulse. Fluorescence parameters were calculated according to Genty *et al.* (1989) and Bilger and Björkman (1990). Each measurement series was replicated on three seedlings.

ETR response curves were assessed two days before seedlings were placed in the growth chamber and following warm temperature/low light conditions (day 17) after the growth chamber treatment (referred to as GCT). The specific areas of the leaves assessed at day 0 for ETR were marked and used subsequently for measurement of F_v/F_m and NPQ, and for ETR at the end of the experiment.

VIS-NIRS data indicated average absorbance of 0.86, 0.89, 0.95 and 0.96 for leaves of NF, F, Sh-NF and Sh-F treatments respectively (Figure 8.7). These values were substituted for the broad estimate of absorbance 0.84 (Bilger and Björkman 1990) which is usually used for caluculation of ETR.

Nutrient analysis

The procedures used were detailed in Chapter 2.

Statistical analysis

Differences in reported variables were analysed as detailed in Chapter 2.

Results

F_v/F_m

F_v/F_m of all treatments decreased ($p < 0.0001$) from optimal (> 0.7) levels before GCT started, to minimum levels 4 days later of 0.25, 0.29, 0.40 and 0.42 for Sh-NF, NF,

Sh-F and F seedlings respectively (Figure 8.1). F_v/F_m of all treatments had increased ($p < 0.005$ and < 0.05 for F and Sh-F, and NF and Sh-NF respectively) by day 13, and then more rapidly ($p < 0.0001$) during the next 4 days of recovery under warm, low light conditions. From day 4 until the conclusion of the experiment on day 17, F_v/F_m of F and Sh-F was higher than that of NF and Sh-NF seedlings ($p < 0.05$ and 0.001 for days 4 to 13 in the growth chamber and days 14-17 under low light).

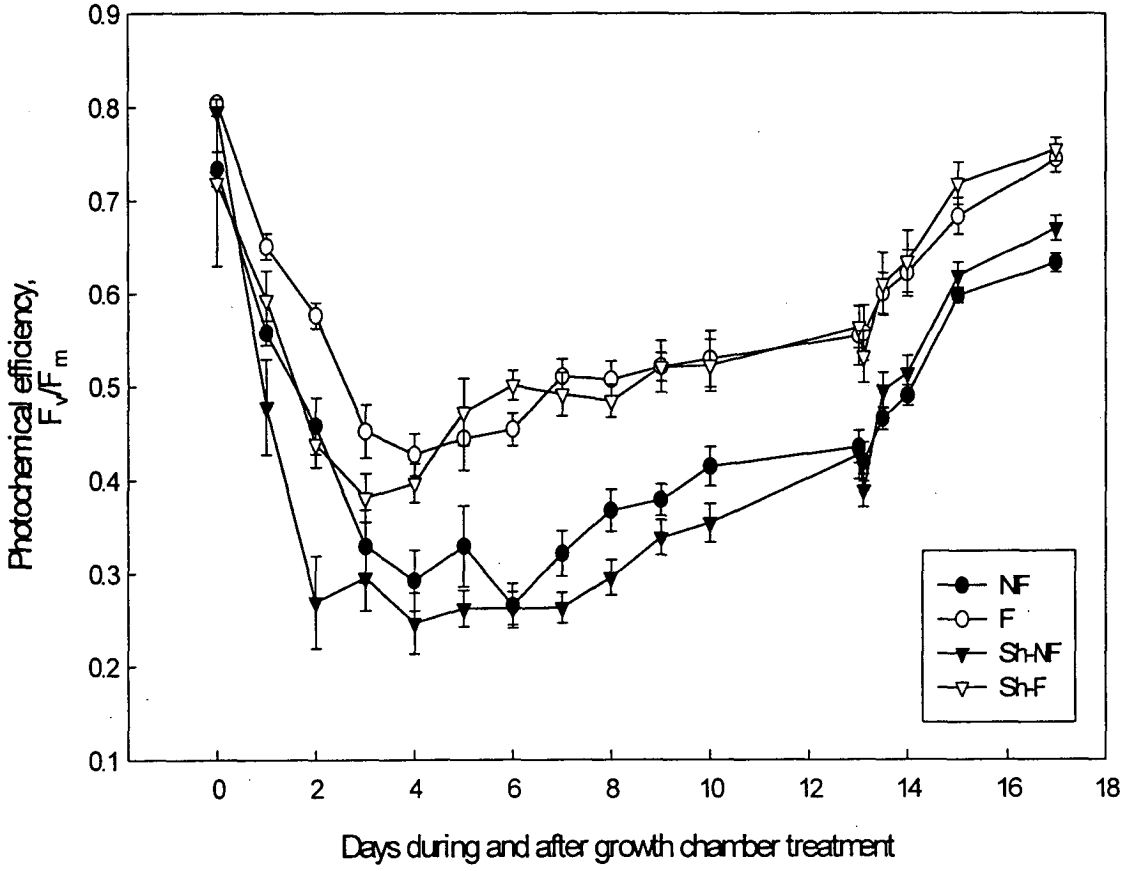


Figure 8.1. Changes in photochemical efficiency (F_v/F_m; dimensionless) with time after exposure to growth chamber conditions (days 0 – 13) and during artificial warm,

low light conditions (days 13 – 18) for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

NPQ

NPQ of NF and F decreased ($p < 0.005$ and $p < 0.05$ respectively) while that of Sh-NF and Sh-F increased ($p < 0.001$ and $p < 0.0001$, respectively) between days 0 and 1 of the GCT (Figure 8.2). Between days 1 to 2, NPQ of all treatments decreased ($p < 0.005$ and 0.0001 for NF and Sh-NF, and F and Sh-F, respectively). After day 2, NPQ levels were relatively constant for all treatments.

At the commencement of GCT, NPQ of NF was significantly higher than F ($p < 0.005$) which in turn was higher than Sh-NF and Sh-F seedlings ($p < 0.0001$) (Figure 8.2). On day 1, NPQ of NF and Sh-NF was higher ($p < 0.05$) than that of F and Sh-F seedlings. After day 4 until the conclusion of the experiment, NPQ of NF and Sh-NF was significantly higher ($p < 0.05$) than NPQ of F and Sh-F seedlings.

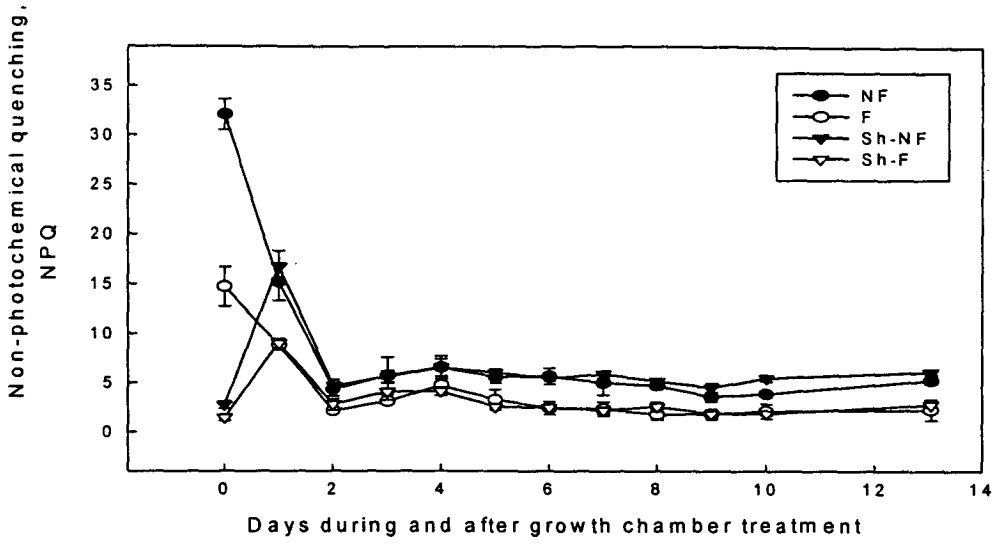


Figure 8.2. Changes in non-photochemical quenching (NPQ; dimensionless) with time after exposure to growth chamber conditions (days 0 – 13) and during artificial warm, low light conditions (days 13 – 18) for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

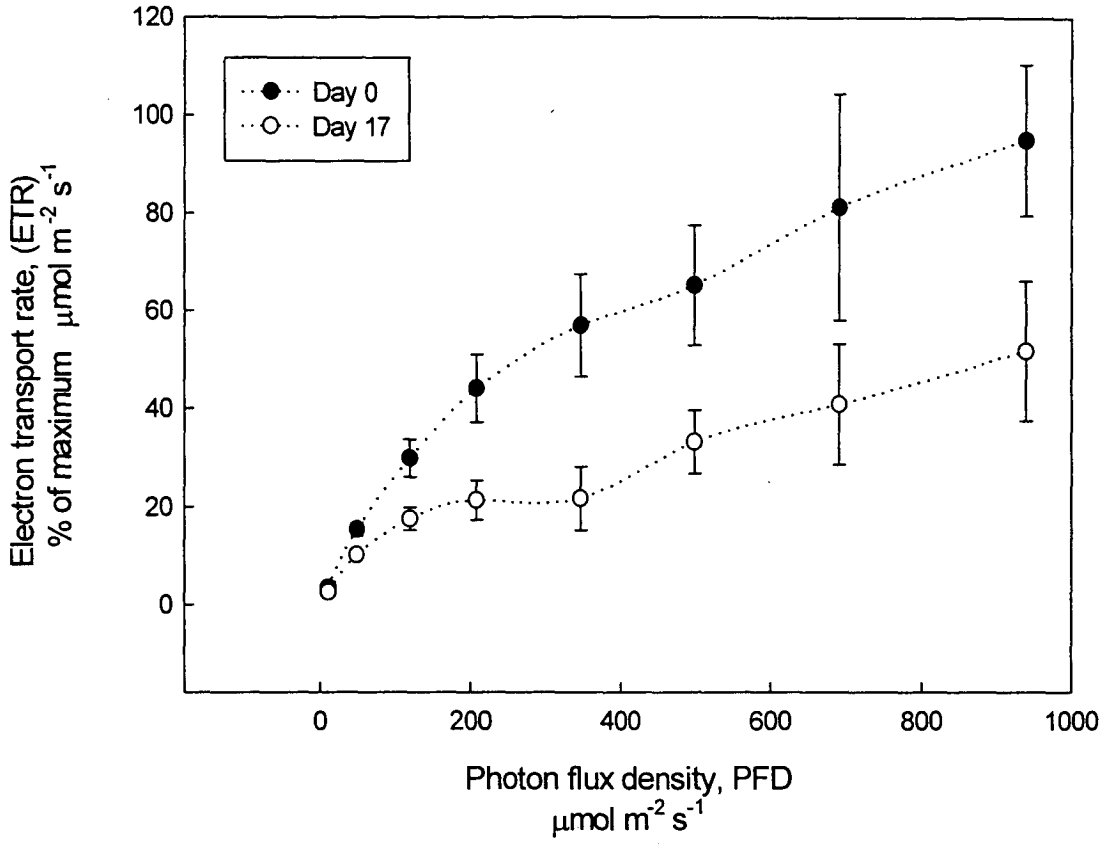


Figure 8.3. Electron transport rate (ETR; μmol m^{-2} s^{-1}) response curves expressed as % of maximum value for NF, F, Sh-NF and Sh-F *E. nitens* seedlings measured before, and after recovery from, growth chamber treatment (GCT). Bars indicate ± standard error.

ETR

ETR (when expressed as % of maximum) was similar between all treatments before and after GCT (Figure 8.3) although actual ETR levels varied greatly both between and within treatments (see Appendix 8). For PFD > 50 μmol m^{-2} s^{-1}, ETR was significantly higher before than after GCT ($p < 0.05$). For PFD > 210 μmol m^{-2} s^{-1}, ETR before GCT was approximately two-fold that measured after GCT.

A+Z/V+A+Z

A+Z/V+A+Z increased rapidly in all treatments between days 0 to 4 after GCT started (Figure 8.4). A+Z/V+A+Z remained constant in NF and F between days 4 and 8 while that of F and Sh-F seedlings decreased ($p < 0.001$). Between days 8 and 12 A+Z/V+A+Z decreased in NF and Sh-NF ($p < 0.01$) but remained constant in F and Sh-F seedlings. A+Z/V+A+Z decreased in all treatments between days 12 to 17 under warm, low-light conditions. Between days 2 and 8, NF and Sh-NF had significantly higher A+Z/V+A+Z than F and Sh-F seedlings ($p < 0.005$).

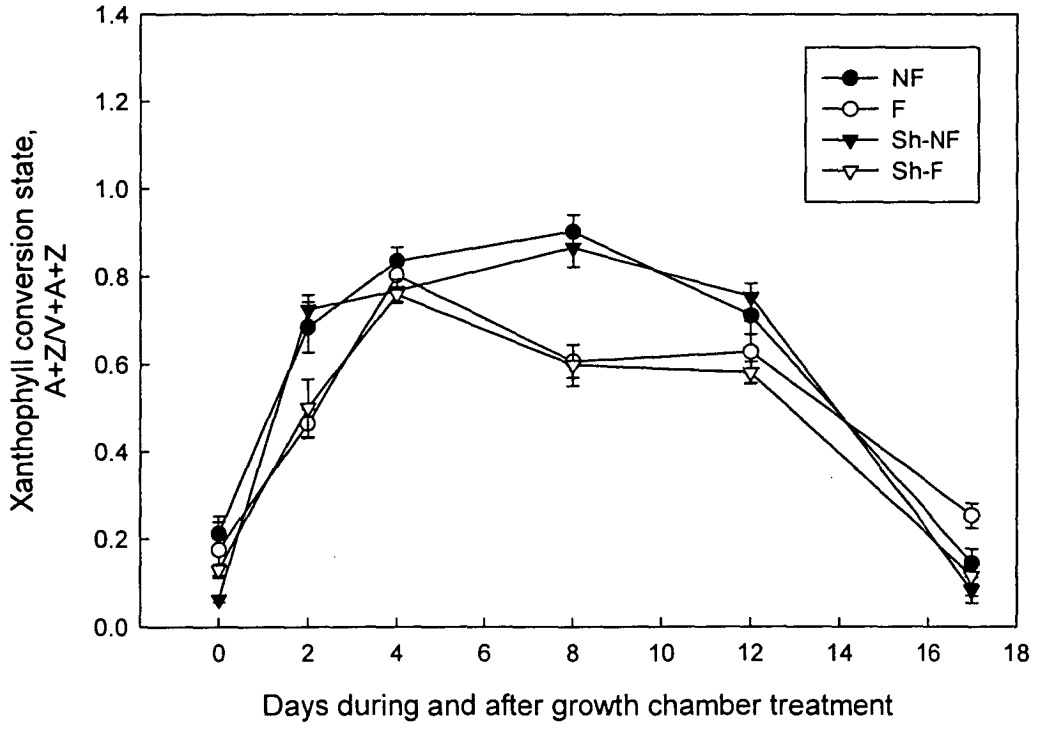


Figure 8.4. Changes in pre-dawn xanthophyll-cycle conversion state (A+Z/V+A+Z; dimensionless) with time after exposure to growth chamber conditions (days 0 – 13) and during artificial warm, low light conditions (days 13 – 18) for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

A+Z/V+A+Z vs. F_v/F_m

A+Z/V+A+Z increased linearly with decreasing F_v/F_m (A+Z/V+A+Z = 0.81 – 0.56* F_v/F_m, $r^2 = 0.84$) (Figure 8.5).

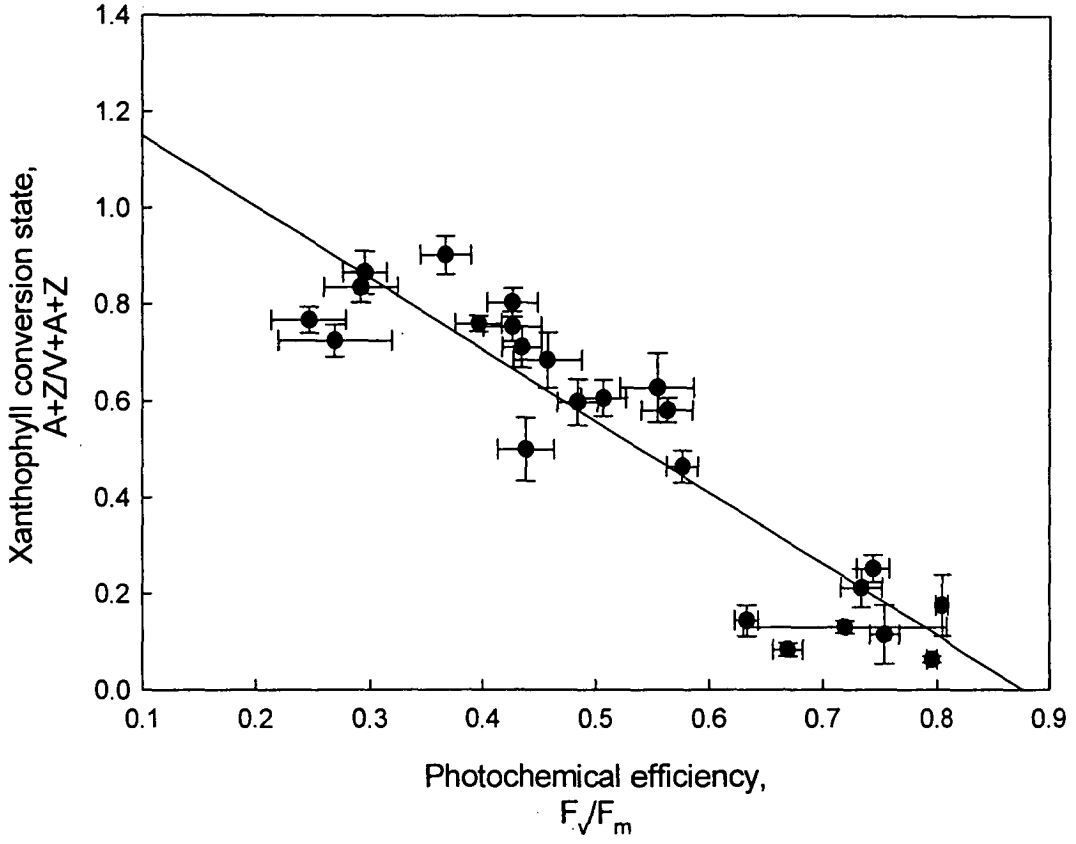


Figure 8.5. The relationship of pre-dawn xanthophyll-cycle conversion state (A+Z/V+A+Z; dimensionless) vs. pre-dawn photochemical efficiency (F_v/F_m; dimensionless) measured throughout exposure to, and recovery from, growth chamber conditions for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Pigment compositions

Chlorophylls and carotenoids

No significant differences between treatments were found in chlorophyll and carotenoid levels between 2 and 12 days after commencement of GCT. The results during this period were averaged and compared to values on day 0.

NF

There were no significant differences in total chlorophylls, chlorophyll *a*:*b*, V+A+Z and ß-carotene per unit chlorophyll (Table 8.2a). Per unit leaf area, carotenopid levels were also similar.

F

Total chlorophylls and chlorophyll *a*:*b* (NS and $p < 0.05$) decreased after day 0 (Table 8.2b). Per unit chlorophyll, lutein and V+A+Z increased ($p < 0.005$, 0.001) after day 0.

Sh-NF

Total chlorophyll decreased, but not significantly ($p < 0.07$) but chlorophyll *a*:*b* was constant between days 0 and 2-12 for Sh-NF (Table 2c). Per unit chlorophyll, V+A+Z increased ($p < 0.005$) and lutein remained constant after day 0. Per unit leaf area, neoxanthin, V+A+Z and ß-carotene decreased ($p < 0.05$, 0.01, 0.09) after day 0.

Sh-F

Total chlorophyll and chlorophyll *a*:*b* decreased ($p < 0.05$) after day 0 (Table 2d). Per unit chlorophyll, lutein and V+A+Z increased ($p < 0.005$, 0.001) after day 0. Per unit leaf area, V+A+Z and ß-carotene increased and decreased ($p < 0.001$ and 0.05), respectively, after day 0.

Table 8.2. Total chlorophyll content (μmol m^{-2}), chlorophyll *a:b* ratio (dimensionless) and carotenoids (the latter expressed in mmol mol^{-1} total chlorophyll^{-1} and μmol m^{-2}) of (a) NF, (b) F, (c) Sh-NF and (d) Sh-F *E. nitens* seedlings before and during and after exposure to growth chamber conditions.

(a) NF	Before GCT	Days 2-12 of GCT
Total chlorophyll (μmol m^{-2})	139 ± 6	135 ± 11
Chl *a:b*	3.80 ± 0.35	3.49 ± 0.57
Carotenoids (mmol mol^{-1} Chl a+b)		
Neoxanthin	56 ± 3	58 ± 12
Lutein	319 ± 7	331 ± 32
V+A+Z	277 ± 22	296 ± 27
β-Carotene	115 ± 7	97 ± 11
Carotenoids (μmol m^{-2})		
Neoxanthin	8 ± 1	8 ± 1
Lutein	44 ± 1	44 ± 4
V+A+Z	38 ± 3	40 ± 4
β-Carotene	16 ± 1	13 ± 1

(b) F	Before GCT	Days 2-12 of GCT
Total chlorophyll (μmol m^{-2})	345 ± 24	278 ± 27
Chl *a:b*	4.02 ± 0.48	3.70 ± 0.81
Carotenoids (mmol mol^{-1} Chl a+b)		
Neoxanthin	50 ± 5	55 ± 7
Lutein	194 ± 14	259 ± 21
V+A+Z	154 ± 8	233 ± 21
β-Carotene	86 ± 7	102 ± 10
Carotenoids (μmol m^{-2})		
Neoxanthin	17 ± 2	14 ± 2
Lutein	67 ± 5	66 ± 6
V+A+Z	53 ± 3	63 ± 5
β-Carotene	29 ± 2	27 ± 3

(c) **Sh-NF**	Before GCT	Days 2-12 of GCT
Total chlorophyll (μmol m^{-2})	274 ± 26	192 ± 12
Chl *a*:*b*	3.47 ± 0.55	3.50 ± 0.37
Carotenoids (mmol mol^{-1} Chl a+b)		
Neoxanthin	55 ± 5	55 ± 5
Lutein	218 ± 25	252 ± 16
V+A+Z	109 ± 10	210 ± 21
β-Carotene	79 ± 10	80 ± 6
Carotenoids (μmol m^{-2})		
Neoxanthin	15 ± 2	11 ± 2
Lutein	59 ± 7	48 ± 6
V+A+Z	29 ± 3	40 ± 5
β-Carotene	21 ± 3	15 ± 3

(d) **Sh-F**	Before GCT	Days 2-12 of GCT
Chl a+b (μmol m^{-2})	391 ± 51	263 ± 34
Chl a:b	4.00 ± 0.89	3.66 ± 0.80
Carotenoids (mmol mol^{-1} Chl a+b)		
Neoxanthin	48 ± 8	52 ± 8
Lutein	178 ± 19	237 ± 25
V+A+Z	98 ± 10	224 ± 18
β-Carotene	85 ± 12	87 ± 12
Carotenoids (μmol m^{-2})		
Neoxanthin	18 ± 3	14 ± 2
Lutein	69 ± 8	62 ± 7
V+A+Z	38 ± 4	58 ± 5
β-Carotene	33 ± 5	23 ± 3

Anthocyanins

Total anthocyanins remained constant between days 0 and 4 and then increased gradually ($p < 0.0001$) between days 4 and 12 after commencement of GCT in F, Sh-NF and Sh-F seedlings (see Plates 8.1a, b, c, d and e). Anthocyanin in NF seedlings

remained constant between days 4 and 12 (Figure 8.6a; see Plates 8.1a, b, c, d and e). Between days 12 and 17, anthocyanin increased ($p < 0.05$) in NF and Sh-NF but remained constant in F and Sh-F seedlings. Anthocyanin was higher in NF seedlings compared to other treatments on days 0, 2 and 4 ($p < 0.01$, 0.001 and 0.005 respectively). Levels were higher in both NF and Sh-NF ($p < 0.05$) compared to Sh-NF and Sh-F seedlings on day 17.

Sideroxylonals

Sideroxylonals decreased in NF ($p < 0.05$) but remained constant in F, Sh-NF and Sh-F seedlings between days 0 and 4 (Figure 8.6b). For the remainder of the experiment sideroxylonals were relatively constant and similar in all treatments. NF had higher levels of sideroxylonals on days 0 and 2 ($p < 0.05$, 0.0001).

Galloylglucoses

Galloylglucoses in NF seedlings decreased ($p < 0.05$) between days 0 to 4 and increased ($p < 0.05$) between days 8 to 12 (Figure 8.6c). Levels in other treatments were relatively constant and similar throughout the experiment. On days 0, 2 and 12, galloylglucoses were higher in NF seedlings compared to other treatments ($p < 0.01$, 0.01 and 0.05 respectively).

Flavonoids

Flavonoids in NF seedlings decreased ($p < 0.05$) between days 0 to 4 and increased ($p < 0.05$) between days 8 to 12 (Figure 8.6d). Levels in other treatments were relatively constant and similar throughout the experiment. On days 0, 2 and 12, flavonoids were

higher in NF seedlings compared to other treatments ($p < 0.05$, 0.01 and 0.05 respectively).

Plate 8.1. NF, F, Sh-NF and Sh-F seedlings (from left to right) before the commencement of growth chamber treatment (GCT) (a) and on days 6 (b), 8 (c), 10 (d) and 12 (e) of GCT. The plate series indicates constant anthocyanin levels in NF seedlings relative to the gradual increase in other treatments between (b) to (e).

a)

b)

c)

d)

e)

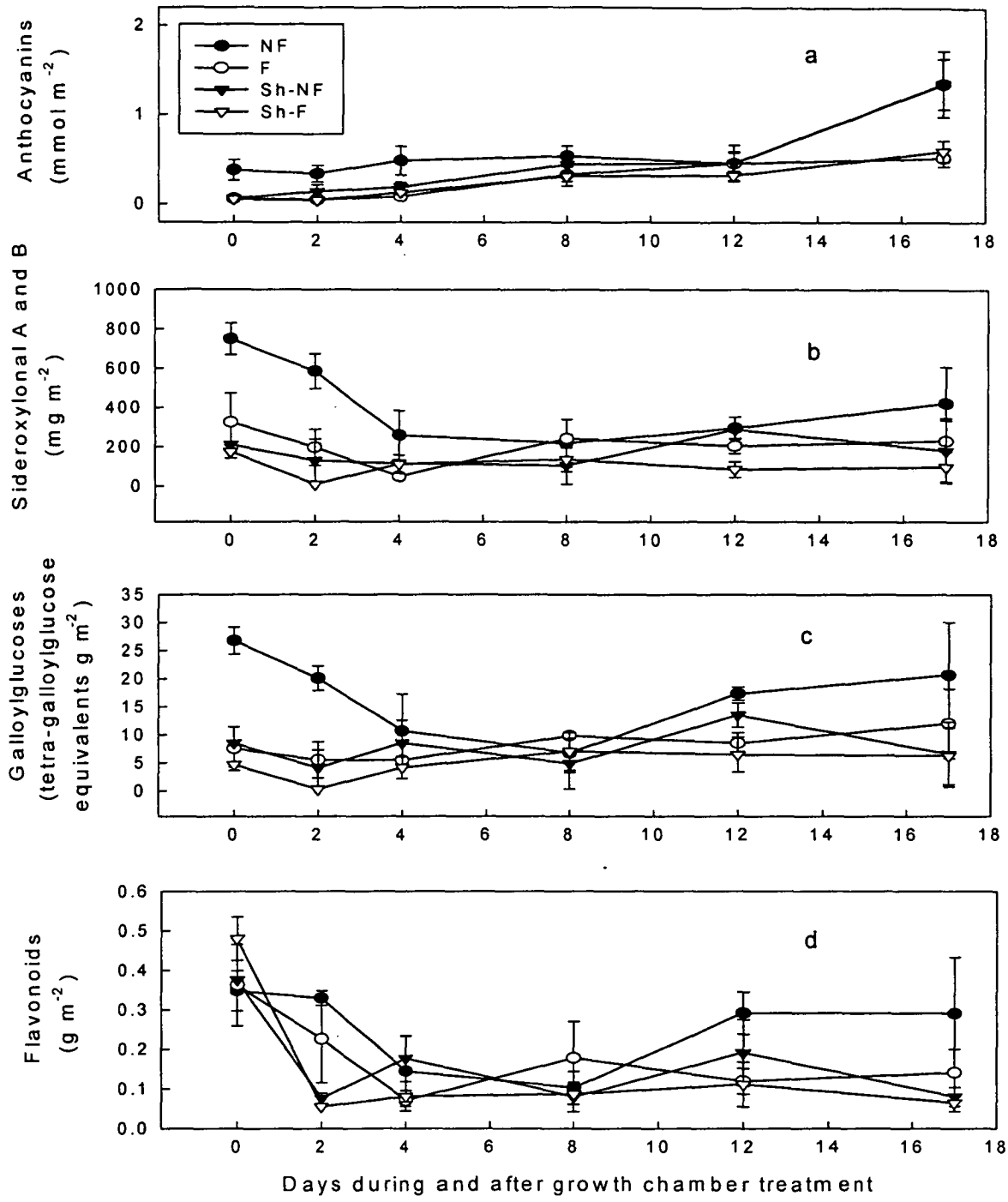


Figure 8.6. Total anthocyanins (mmol m^{-2}) (a), sideroxylonal A and B (mg m^{-2}) (b), total galloylglucoses (tetra-galloylglucose equivalents g m^{-2}) (c), and total flavonoids (g m^{-2}) (d) in NF, F, Sh-NF and Sh-F *E. nitens* seedlings throughout exposure to (days 0 – 12), and after recovery from (day 17), growth chamber treatment (GCT).

Visible/near infra red spectroscopy (Vis-NIRS)

All treatments show increased absorption between 510 and 580 nm after commencement of GCT (Figure 8.7). F, Sh-NF and Sh-F had decreased absorption between 600 and 750 nm compared to NF seedlings.

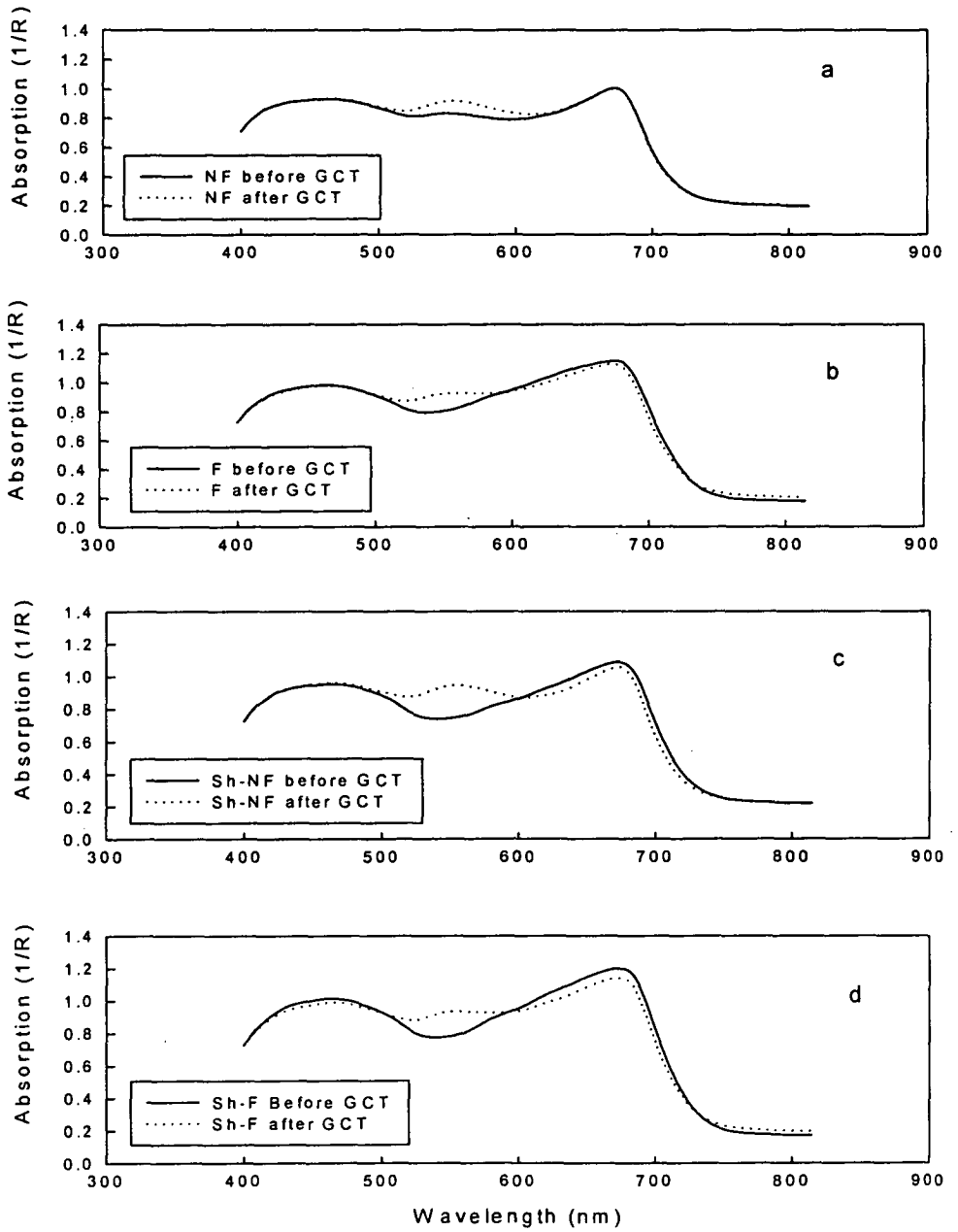


Figure 8.7. Near infrared spectra (VIS-NIRS) of approximately 30 leaves of *E. nitens* seedlings sampled before and 17 days after growth chamber treatment (GCT).

Nutrient analysis

Total N

Total N decreased significantly ($p < 0.0001$) in fertilised treatments between days 0 and 17 but remained similar in non-fertilised treatments (Figure 8.8a). Fertilised had significantly higher ($p < 0.0001$) total N compared to non-fertilised treatments.

Soluble N

Soluble N decreased in F and Sh-F ($p < 0.001$, NS respectively) seedlings between days 0 and 7 but remained similar in non-fertilised treatments (Figure 8.8b). Fertilised were significantly higher ($p < 0.05$) in soluble N than non-fertilised treatments.

Nucleic acid N

Nucleic acid N decreased in F and Sh-F ($p < 0.05$, NS respectively) seedlings between days 0 and 7 but remained similar in non-fertilised treatments (Figure 8.8c). Fertilised were significantly higher ($p < 0.05$) in nucleic acid N than non-fertilised treatments.

Protein N

Protein N comprised > 90 % of total N (Figure 8.8d). It followed the same patterns of change as that of total N.

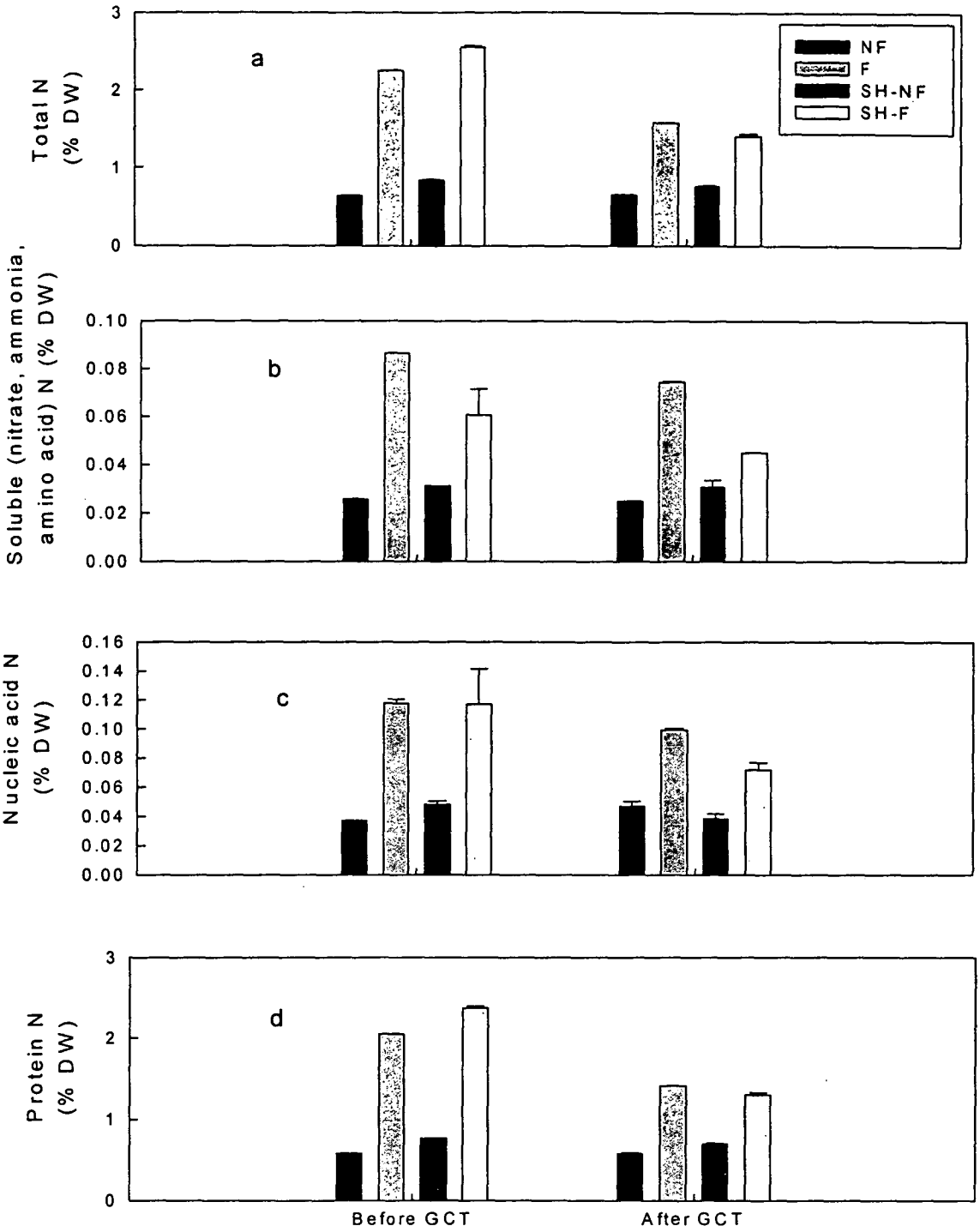


Figure 8.8. Changes in (a) total, (b) soluble (nitrate, ammonia and amino acid), (c) nucleic acid and (d) protein nitrogen (% fresh weight) before and 17 days after the growth chamber treatment (GCT) commenced for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Total P

Total P significantly decreased ($p < 0.001$) in fertilised treatments between days 0 and 7 but remained similar in non-fertilised treatments (Figure 8.9a). Total P was higher in fertilised compared to non-fertilised treatments except between Sh-NF and Sh-F seedlings on day 17.

Sugar phosphate

Sugar P decreased in all treatments ($p < 0.05$, 0.0001, NS and NS for NF, F, Sh-NF and Sh-F respectively) between days 0 and 17 (Figure 8.9b). Non-fertilised had higher levels of sugar P ($p < 0.05$, 0.001 for non-shaded and shaded seedlings respectively) than fertilised treatments on day 17.

Nucleic acid P

Nucleic acid P decreased ($p < 0.05$, NS for F and Sh-F respectively) in fertilised but remained similar in non-fertilised treatments between days 0 and 17 (Figure 8.9c). Nucleic acid P was higher ($p < 0.05$) in fertilised compared to non-fertilised treatments during both measurement periods.

Inorganic P (P_i)

P_i decreased ($p < 0.05$) in Sh-F seedlings between days 0 and 17 (Figure 8.9d). No changes were observed in other treatments. Non fertilised had higher levels of P_I ($p < 0.05$) relative to fertilised treatments on day 17.

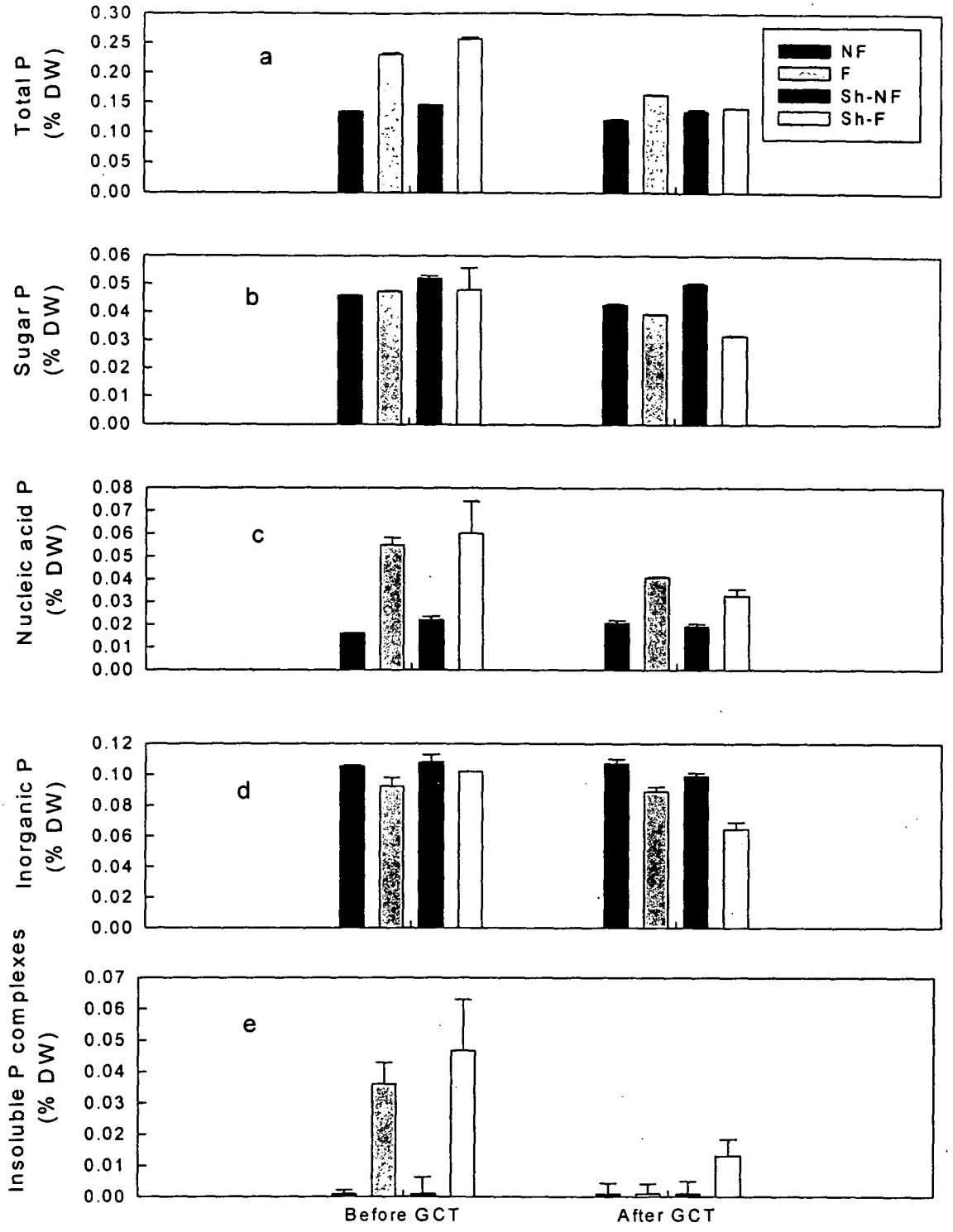


Figure 8.9. Changes in (a) total, (b) nucleic acid, (c) phosphate and (d) inorganic phosphorus and (e) insoluble P complexes (% fresh weight) before and 17 days after the growth chamber treatment (GCT) commenced for NF, F, Sh-NF and Sh-F *E. nitens* seedlings. Bars indicate ± standard error.

Insoluble P complexes

Insoluble P complexes decreased between days 0 and 17 in fertilised ($p < 0.05$) but not non-fertilised treatments (Figure 8.9e). Fertilised had significantly higher insoluble P complexes than non-fertilised treatments on day 0 ($p < 0.01$). On day 17 only, Sh-F seedlings had higher levels than other treatments ($p < 0.05$).

Discussion

Major findings

This experiment has shown that anthocyanins absorb visible light between 400 and 600 nm. Transfer of seedlings from shaded (470) and non-shaded (1460) μmol m^{-2} s^{-1} and 21.0 °C conditions in a nursery to 580 μmol m^{-2} s^{-1} and 14.4 °C in a growth chamber rapidly induced sustained xanthophyll cycle activity (measured as decreased F_v/F_m and increased A+Z/V+A+Z) and adjustment of chlorophyll and lutein and xanthophyll per unit chlorophyll pools. The kinetics of anthocyanin synthesis indicated acclimation 8 days after commencement of growth chamber treatment (GCT). The kinetics of chlorophyll and carotenoid synthesis indicated acclimation 2 days after commencement of GCT. Depressed ETR response curves after GCT commenced were the net effect of decreased light absorption, increased light energy dissipation and increased light attenuation. These were causally linked to levels of chlorophyll, increased xanthophyll pools (n.b. the xanthophyll cycle was relaxed ensuring sustained activity did not dissipate excess light energy) and increased levels of anthocyanin, respectively. The similarity of ETR response between all treatments given widely ranging pigment levels (other than anthocyanins) implies light attenuation by anthocyanins. Changes in levels of sideroxylonals were similar to those of galloylglucoses and flavonoids so that they may be acting as antioxidants

also. The requirement for these antioxidants appeared to be higher during periods of increased light utilisation when greater levels of reactive oxygen species exist, compared to periods of increased dissipation.

F_v/F_m, A+Z/V+A+Z, chlorophyll, carotenoid and anthocyanin compositions

The effects of photoinhibition following transfer of the seedlings from the nursery to the growth chamber were immediate. 'Pre-dawn' F_v/F_m steadily decreased from optimal levels in all treatments to extremely low levels within four days. An overriding influence of low temperature, rather than irradiance level, in causing this cold-induced photoinhibition was indicated. The irradiance in the growth chamber was less than half that incident in the nursery indicating that the decreased day and night time leaf temperatures following transfer induced photoinhibition. The decreases in F_v/F_m were more rapid in shaded than non-shaded treatments. This was consistent with the lower levels of total chlorophyll and larger pools of lutein and xanthophyll per unit chlorophyll in non-shaded compared to shaded seedlings before transfer to the GCT and the different balance of pigments required for the absorption, utilisation and dissipation of light between these treatments.

These decreases in F_v/F_m were associated with an increased conversion state of the xanthophyll cycle. Higher A+Z/V+A+Z in non-fertilised than fertilised treatments was measured two days after the commencement of GCT. This was not reflected in differences in F_v/F_m between these treatments. This may be because different individual seedlings were used for xanthophyll pigment analysis and measurement of F_v/F_m or more likely, because mechanisms other than the xanthophyll cycle were involved in the dissipation of excess light (see Chapters 5 and 6). Four days after the

commencement of GCT, F_v/F_m of all treatments began to increase at similar rates, suggesting acclimation to the growth chamber conditions. This was expressed as decreased levels of total chlorophyll and chlorophyll *a*:*b* and increased V+A+Z and lutein per unit chlorophyll, particularly in fertilised treatments. Such acclimation (ie. the combined effects of decreased total chlorophyll and/or increased V+A+Z to increase V+A+Z:total chlorophyll overall) is a common response to photoinhibitory conditions (Adams and Demmig-Adams 1994; 1995; Adams *et al.* 1995; Ottander *et al.* 1996; Verhoeven *et al.* 1996; Adams and Barker 1998; Logan *et al.* 1998) although recovery of F_v/F_m under photoinhibitory conditions, as opposed to under artificial warm, low light conditions, has not been reported previously.

Eight days after commencement of GCT, A+Z/V+A+Z was lower in fertilised than non-fertilised treatments. The xanthophyll conversion state had also decreased significantly after day 4 in the fertilised treatment. Similarly there were larger changes in the levels of chlorophylls and xanthophylls during acclimation (between days 0 and 2), and higher minimum F_v/F_m and NPQ levels, in fertilised than non-fertilised treatments. A+Z/V+A+Z decreased significantly in non-fertilised treatments between days 8 and 12 and then was similar to levels in fertilised treatments. However F_v/F_m remained significantly lower in non-fertilised than fertilised treatments at this time. Different rates of induction of mechanisms which dissipate excess light energy other than the xanthophyll cycle, such as D1 metabolism (Thiele *et al.* 1996; Shang and Feirabend 1998; Forster *et al.* 1999; Darkó *et al.* 2000) and LHC re-organisation (Ottander *et al.* 1995; Giardi *et al.* 1996, 1997; Geiken *et al.* 1998; Matoo *et al.* 1999) may explain this divergence between the fertilised and non-fertilised treatments. An alternate explanation lies in the synthesis of anthocyanins.

Anthocyanin levels gradually increased in the non-fertilised treatments and, through increased light attenuation, may have protected lower cellular layers, allowing gradual recovery of A+Z/V+A+Z to levels observed in fertilised seedlings. This argument is consistent with the inverse relationship between anthocyanin levels per unit chlorophyll and the levels of total chlorophyll and xanthophyll pool per unit chlorophyll after day 8. Thus, anthocyanin synthesis may reinforce acclimation to photoinhibition which is first driven by changes in levels of chlorophyll and carotenoids. However, if anthocyanins attenuate light, A+Z/V+A+Z for a given PFD would be lower once appreciable levels of anthocyanin were synthesised which was not observed. This is consistent with the conclusion (also drawn in Chapters 5 and 6) that the maximum capacity of the xanthophyll cycle to dissipate excess light energy is reached well below levels of non-photochemical quenching measured in *E. nitens* foliage.

Electron transport rate (ETR)

ETR was approximately 50% less in the same leaves after than before the commencement of GCT, regardless of treatment (differences between replicates give the false impression that leaves were not light saturated). This was despite large differences in chlorophyll, carotenoid and anthocyanin contents between treatments. Levels of A+Z/V+A+Z and F_v/F_m indicated that the xanthophyll cycle at the time of measurement of ETR (day 17) was fully relaxed, and that the mechanisms of altered D1 protein metabolism and LHC re-organisation would have reverted to conditions prevailing in the nursery (Ottander *et al.* 1995). Thus depressed ETRs were not due to processes that affect excess light energy dissipation. Anthocyanin levels were inversely related to total chlorophyll levels. Thus the initial decrease in total

chlorophyll levels and the later increase in anthocyanin levels may combine to cause the decrease in ETR. This process occurs within the dictates of the initial pigment levels and seedling nutrition of each treatment: in Sh-F seedlings there was considerable scope to decrease levels of total chlorophyll and increase levels of xanthophylls. This was observed and effectively re-balanced light energy absorption and dissipation to the changed conditions. In NF seedlings, there was less scope for such changes and anthocyanin levels were increased to re-balance absorption through light attenuation. F and Sh-NF seedlings fell within these two boundaries of response.

Visible/near infra red spectroscopy (VIS-NIRS)

VIS-NIRS measures the net effect of changes in pigment levels. In the order of magnitude, F > Sh-NF > Sh-F> NF, there was decreased absorption between 600 and 740 nm 17, compared to 0, days after the commencement of GCT. This is consistent with observed relative amounts of total chlorophyll oxidised in treatments (see Tables 8.2a, b, c, and d) during the photooxidation of chlorophyll acclimation process (Matoo *et al.* 1999). VIS-NIRS also supported the hypothesis that anthocyanins attenuate incident light during photoinhibition. All treatments exhibited increased absorption between 510 and 580 nm, consistent with absorption by increased levels of foliar anthocyanin (Pietrini and Massacci 1998; Neill and Gould 1999). Greater differences in light absorption between 0 and 17 days after GCT within shaded treatments concurs with the fact that before the commencement of GCT, anthocyanin levels were close to zero relative to pre-GCT levels of non-shaded treatments. Light absorption by anthocyanin between 400-600 nm has been attributed to the modulation of light available to the chloroplasts, thus providing a photoprotective role (Pietrini and Massacci 1998).

Galloyolglucoses, sideroxylonals, flavonoids and NPQ

The relative decreases in galloyolglucose, sideroxylonal and flavonoid levels in NF seedlings between commencement and day 4 of GCT were similar. This was unexpected, given the previously reported roles of galloylglucoses and flavonoids as antioxidants (Hodnick *et al.* 1988; Ariga and Hamano 1990; Okamura *et al.* 1993b; Hagerman *et al.* 1998; Gardner *et al.* 1999) and the observation in Chapter 7 where galloyolglucoses and flavonoids increased in response to the severity of photoinhibition. Similar kinetics between galloyolglucoses, flavonoids and sideroxylonals observed here suggest sideroxylonals may play an antioxidant role also, but under conditions of high rates of photosynthesis rather than photoinhibition. Sideroxylonals have, in common with recognised antioxidants, high levels of conjugation and hydroxylation (Eschler and Foley 1999). If sideroxylonals can act as physiological antioxidants, their function as compounds deterring herbivory may indicate their exploitation in a dual role by plants. It has been suggested that domestication of *Olea europaea* L. var. europaea, which has focused on maximising growth rate at the expense of foliar 'tannin' production, may increase its susceptibility to herbivory (Massei and Hartley 2000). Recently it has been shown that a complex group of formylated phloroglucinol compounds, though not sideroxylonals, are present in *E. globulus* (Eschler *et al.* 2000), a species susceptible to photoinhibition.

Given their capacity as antioxidants, increased levels of galloyolglucoses and flavonoids in all treatments was anticipated following transfer to GCT. A possible explanation may be based on change in NPQ. NPQ was very high in NF seedlings prior to transfer to GCT. NPQ rapidly decreased to low levels by day 2, a change which is related to that observed with levels of antioxidants. The decrease in

antioxidant levels closely paralleled that of F_v/F_m over the same period. Under nursery conditions of mild temperature no sustained xanthophyll activity was measured, thus a greater production of reactive oxygen species may have occurred, requiring greater amounts of antioxidants for effective quenching. Conversely, under GCT which had relatively low temperature and irradiance, sustained xanthophyll cycle activity and possibly altered D1 protein metabolism and/or LHC re-organisation effectively dissipated excess light energy and thus decreased the requirement for high levels of antioxidants.

Nutrient analysis

Total N and P decreased in fertilised but not non-fertilised treatments between 0 and 17 days after GCT. This was consistent with the results observed between planting and first field measurement reported in Chapters 4 and 5 and in transplanted *Picea glauca* (McAlister and Timmer 1998) and *Picea mariana* (Kim *et al.* 1999) and indicates translocation of N and P from shoots to roots in the fertilised treatments. In the growth chamber, no additional nutrients were applied to fertilised seedlings. Thus nutrients remaining in the potting mix following nursery application would have been rapidly leached. The potting mix was predominately pine bark which was free draining once moist and had little inherent capacity to store and supply nutrients.

Changes in the chemical fractions of N and P also support translocation of N and P. Protein N and inorganic P, the major storage forms of N and P respectively (Chapin and Kedrowski 1983), accounted for the great majority of decrease of total N and P. In comparison, relatively small decreases in nucleic acid N and P and soluble N occurred which were indicative of decreased metabolic activity and N uptake,

respectively. These effects were probably because of the low temperature growing conditions within the growth chamber and cessation of fertilisation during the experiment.

Conclusion

Anthocyanins absorb radiation between 400 and 600 nm. This provides a mechanism that can attenuate light according to levels of utilisation and dissipation. The rapid process of photooxidation decreases chlorophyll levels to balance light absorption with its utilisation and dissipation within days. These processes combine to facilitate the recovery of PSII efficiency under conditions of cold-induced photoinhibition. The distinct kinetics of xanthophyll cycle conversion state on the one hand and PSII efficiency on the other provided further suppport to the conclusion that mechanisms other than the xanthophyll cycle can quench excess light energy (see Chapters 5, 6 and 7). The kinetics of flavonoid and galloylglucose synthesis indicated that the quenching of reactive species was a greater requirement under conditions of high light utilisation compared to conditions of sustained excess energy dissipation. The parallel changes in sideroxylonals to flavonoids and galloylglucoses indicates that sideroxylonals may act also as antioxidants.

Nutrients were possibly translocated to the root from stored sources of N (protein N) and P (inorganic P) in the shoot after planting.

In this experiment rapidly induced cold-induced photoinhibition required adjustment of mechanisms of acclimation, including that of antioxidant levels. These adjustments prevented photobleaching. However, if rapidly induced conditions of cold-induced

photoinhibition are more severe, the capacity of mechanisms of acclimation can be superceded and photobleaching results. The occurrence of these conditions are the subject of the following chapter.

Chapter 9. Cold-induced photoinhibition and photobleaching reduces growth of *Eucalyptus globulus* seedlings

Introduction

Background observations and hypothesis

In Tasmania, *E. globulus* seed is usually sown in late February and March to allow for good establishment, growth and acclimation of seedlings before winter (I.C. Ravenwood, pers comm). Seed was germinated on 25 May 1998 (unusually late in the season for *E. globulus*) at the Somerset nursery of North Forest Products. Unintentionally, shadecloth covers were not in place to shelter unhardened seven-week-old seedlings. The seedlings were exposed to freezing overnight temperatures and bright sunlight the following morning (10 July 1998). Approximately 3 days after this event, leaf tissue damage expressed as visible yellowing was observed. Only certain areas of the tray bed were affected and it was noted that there were 'strips' of undamaged seedlings. These strips were approximately 4 m apart, the same distance apart as the steel frames that support the shadecloth. These frames cast a moving band of shade onto the tray bed under clear sky conditions. The majority of damage was to expanded leaves rather than growing tips. It was hypothesised

- that photoinhibition had caused the tissue damage (ie. photodamage) and not frost, as growing tips are more susceptible to frost damage than mature leaves (Steponkus 1984).

Twelve days after the frost event, chlorophyll fluorescence (yield and NPQ) and leaf area with symptoms of photobleaching were estimated in both damaged and

undamaged seedling strips. The precise period during which tissue damage occurred was investigated and the effects on subsequent seedling performance assessed.

Materials and Methods

Plant Material

Seedlings of *E. globulus* were raised from seedlot G2490 (North Forest Products Pty. Ltd. improved seed) in 115 cm^3 plugs in the North Forest Products' Somerset nursery. Seed was germinated on 25 May, 1998 and germinated in a glasshouse at approximately 20 °C. After 4 weeks, the seedlings were moved to an open area sheltered by movable 50% shadecloth covers, but otherwise exposed to ambient conditions. Seedling growth was supported by feeding with Aquasol® every 10 days (Solution concentration 1100 – 1500 mS).

Environment

Air temperature (°C) at bench height (800 mm) and total incident shortwave radiation (W m^{-2}) on a horizontal surface were measured by a nursery climate control and weather station system ('PlantPlan', Levin, NZ). The area of the tray bed shaded by the steel support frames was recorded every half hour from 0800 h until 1500 h.

Chlorophyll Fluorescence

Measurements of steady state fluorescence were conducted as detailed in Chapter 2 and made at hourly intervals from 0900-1500 h on 22 July 1998 (the first cloud-free day since the 10 July). Incident photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}) perpendicular to the surface of the leaf for each measurement was measured by a quantum sensor attached to the leaf clamp of the PAM-2000. Fully expanded leaves

of three randomly selected seedlings from each of the damaged and undamaged areas (respectively the exposed and shaded area between 1000-1030 h on 10 July) were measured. All leaves selected for measurement were intact. In the damaged treatment, only green areas of the leaves were used where visible yellowing did not exceed 30%.

Photobleaching and height growth

Percent leaf area showing symptoms of photobleaching was estimated visually (in increments of 10%) on ten randomly selected seedlings from the damaged and undamaged strips. Seedling height on 60 randomly selected seedlings within each strip was measured on the 22 September.

Statistical analysis

Differences in chlorophyll fluorescence variables, percentage leaf area damage and height were analysed as detailed in Chapter 2.

Results

Environment

Diurnal patterns of total incident radiation (irradiance) on 10 and 22 July were similar and generally uninterrupted by cloud (Figures 9.1a, b). Sunlight was first recorded at 0750 h. Irradiance peaked at 250 W m^{-2} at 1200 h on both days. On 10 July, air temperature was 0.5 °C at 0750 h and reached 10 °C at 1115 h (Figure 9.1c). On 22 July, air temperature was 3 °C at 0750 h and reached 10 °C at 0940 h (Figure 9.1d).

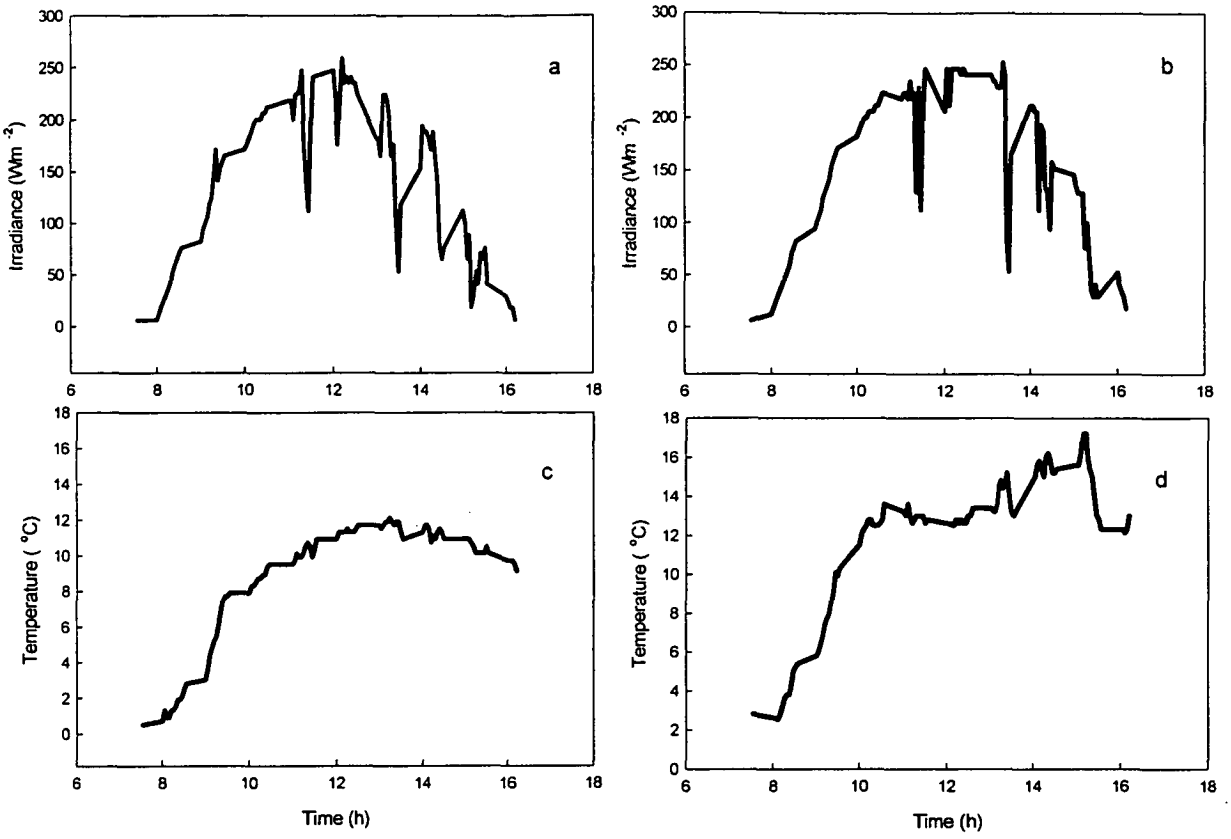


Figure 9.1. Diurnal changes in total incident shortwave radiation (W m^{-2}) incident at the Somerset nursery on 10 July 1998 (a) and 22 July 1998 (b); and air temperature (°C) profile at tray bed height on 10 July 1998 (c) and 22 July 1998 (d).

The shadow caused by the steel support frame coincided with the area of undamaged seedlings between 1000 and 1030 h. During this period on 10 July, air temperature was 7.8-8.5 °C and irradiance 170-200 W m^{-2}.

Maximum incident PAR at the leaf surfaces varied between 650 - 830 μmol m^{-2} s^{-1}. The shadow reduced PAR to between 45 – 65 μmol m^{-2} s^{-1} (Figure 9.2).

Plate 9.1. Shaded and non-shaded seedling strips approximately 10.20 am on 22 July

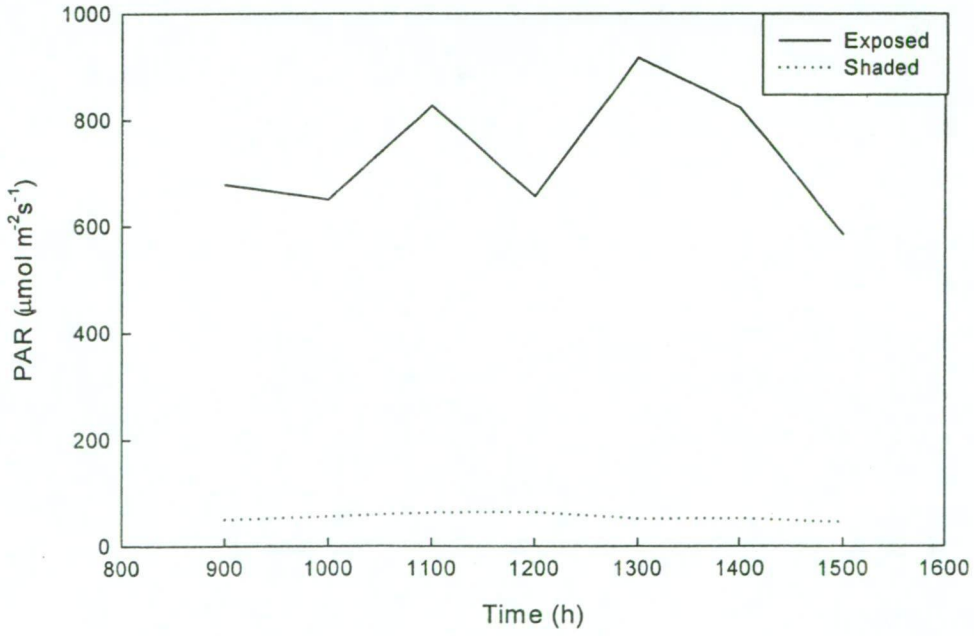


Figure 9.2. Diurnal changes in photosynthetically active radiation (PAR, µmol m^{-2} s^{-1}) incident on leaves of *E. globulus* seedlings on the 22 July 1998.

Chlorophyll Fluorescence

Quantum yields were significantly higher ($p < 0.001$) in undamaged compared to damaged seedlings but remained relatively constant within treatments throughout the day (Figure 9.3a). In the undamaged seedlings, yields varied between 0.4 and 0.5, and in the damaged seedlings between 0.1 and 0.2.

Non-photochemical quenching (NPQ) was significantly higher ($p< 0.05$) in damaged compared to undamaged seedlings (Figure 9.3b). NPQ was near zero in both treatments at 0900 h but significantly higher ($p= 0.042$) in damaged compared to undamaged seedlings. NPQ increased rapidly over the next hour to about 12 and 7 in damaged and undamaged seedlings, respectively. NPQ continued to increase between 1100 h and 1200 h in damaged seedlings to reach a maximum of 16.5. After 1200 h, NPQ decreased gradually to 12 and 0 at 1500 h in damaged and undamaged seedlings, respectively.

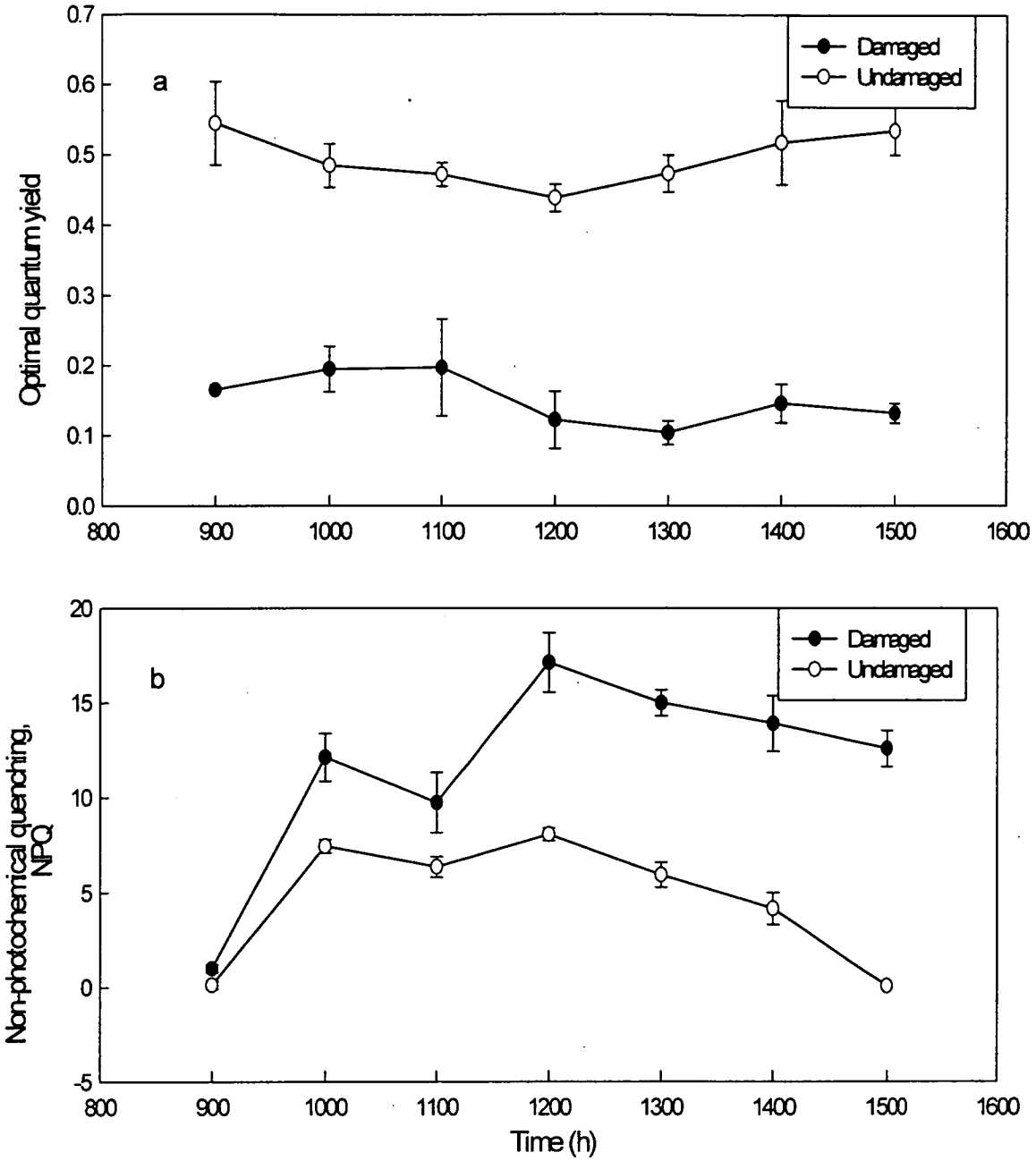


Figure 9.3. Diurnal changes in optimal quantum yield (a) and non-photochemical quenching (NPQ) (b) of *E. globulus* leaves on the 22 July 1998. All variables are dimensionless.

Leaf area damage and seedling height growth

Percent leaf area bleached was significantly greater ($p < 0.01$) in damaged (29%) compared to undamaged (11%) seedlings (Figure 9.4). Two months after the frost event, undamaged seedlings had significantly ($p < 0.001$) greater height (12.5 cm) compared to damaged seedlings (8.0 cm).

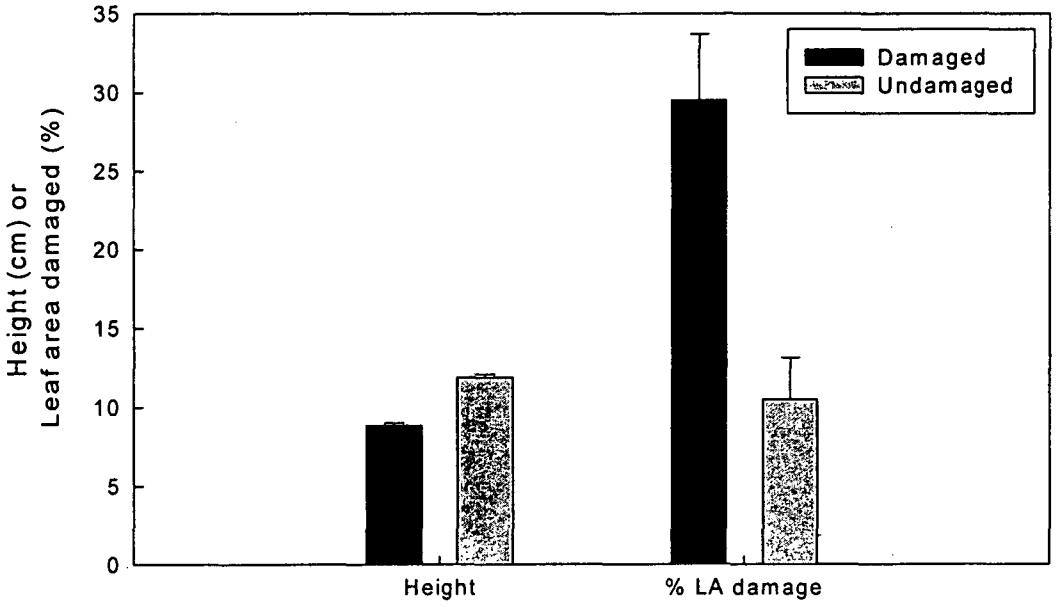


Figure 9.4. % leaf area damage estimated on 22 July 1998 and height growth (cm) measured on 22 September 1998, for damaged and undamaged seedling strips at the Somerset nursery.

Discussion

Major findings

Photoinhibition of unhardened *E. globulus* seedlings in a nursery has been demonstrated. In the absence of shade, levels of photobleaching that were sufficient to significantly reduce subsequent height growth.

This conclusion was reached because the photobleaching was in clearly defined strips of seedlings interspersed with undamaged strips. These strips coincided with the areas shaded by the steel support frames between 1000 and 1030 h on 22 July (Plate 9.1). As this was only 12 days after the event, it can be assumed that the strips were shaded at similar times on both days. Thus only a narrow window of the combined variables of temperature and radiation was required for extensive photodamage to occur. The photobleaching occurred as a consequence of the combined effects of high incident PAR (700-800 μmol $m^{-2}s^{-1}$) and low temperature (8 °C) between 1000 and 1030 h on 10 July 1998. From the comparison of temperature profiles of 10 and 22 July it appears that if the air temperature is <10 °C young seedlings may be susceptible to photobleaching at the highest incident PAR levels associated with winter sunlight.

Cold-induced photoinhibition - irradiance

Irradiance increased steadily from 0800 to 1130 h at the nursery but PAR at the leaf surface was consistently high, between 650 and 850 μmol m^{-2} s^{-1}. Leaves oriented towards the sun in the morning maximised their interception of incident light and appeared to have higher levels of photobleaching than other leaves. There was also evidence of some photobleaching (10%) of such leaves in the areas shaded between

1000 and 1030 h indicating that the period of physiological stress had extended beyond this time period.

Cold-induced photoinhibition – air temperature and chlorophyll fluorescence

The weather station records indicate that air temperatures prevailing between 11 and 22 July were considerably higher than those on 10 July. The differences between treatments measured on 22 July were therefore a manifestation of the severe photoinhibitory conditions present on 10 July. Cold-induced photoinhibition is more commonly associated with cooler climates (Öquist 1987; Ögren and Sjöstrom 1990). If severe, it is associated with the loss of chlorophyll and photobleaching (Oberhuber and Bauer 1991; Haldimann 1999). Visible yellowing of the leaves of the *E. globulus* seedlings leaves was indicative of severe photobleaching. However, areas of leaves that showed no visible damage on 22 July may have been under physiological stress since the 10 July. The low optimal quantum yields in the damaged compared to the undamaged seedlings indicated that this was the case. The low yields led to increased levels of excess light energy and a greater requirement for NPQ which was greater for damaged than undamaged seedlings. In general, the diurnal profile of NPQ paralleled the rise and fall of incident irradiance: the lower levels at 1100 h coincided with a short period of cloud cover. The rapid increase of NPQ between 0900 and 1000 h is consistent with the steep rise in available light that occurred during the period and with seedlings being most vulnerable to photoinhibition between 1000 and 1030 h under the conditions prevailing on 10 July 1998. Yield and NPQ of undamaged seedlings were similar to those found on other healthy plants (Bilger *et al.* 1995; Massacci *et al.* 1995; Barker *et al.* 1998). In contrast, NPQ of the damaged seedlings was high compared to levels reported for cold-induced photoinhibition of *E. nitens*

seedlings (Warren *et al.* 1998). However Warren *et al.* (1998) only exposed seedlings to low temperature for around 55 min. It has been demonstrated that longer periods of exposure to low temperature increase the incidence and extent of photobleaching (Ögren and Sjöstrom 1990).

Susceptibility to cold-induced photoinhibition

Immature leaves have been found to have lower chlorophyll levels, lower rates of photosynthesis and higher levels of photoinhibition compared to mature foliage (Krause *et al.* 1995; Dodd *et al.* 1998). Young seedlings of *E. globulus* and *E. nitens* planted into the field have similar characteristics as well as lower levels of carotenoids (relative to mature foliage) to dissipate excess energy (Close *et al.* 1999). The very high levels of NPQ observed in the *E. globulus* seedlings in the nursery were consistent with physiologically immature leaves being highly susceptible to photoinhibition. Similarly high values of NPQ were found in *E. nitens* and reported in Chapters 4 and 7.

Cold-induced photoinhibition, photobleaching and growth

When mechanisms for energy dissipation are superseded, reactive oxygen species are produced (Niyogi 1999). This gives rise to photobleaching (Asada 1992). 29% of leaf area was visibly damaged in the seedlings exposed to high irradiance on 10 July and the fluorescence variables indicated that the undamaged foliage was under physiological stress on 22 July. Thus, photoinhibition was responsible for the significant reduction in height growth of the damaged compared to undamaged seedlings (expressed two months after the photodamage event). Quantitative reductions in growth following cold-induced photoinhibition have been reported

previously for *Brassica napus* (Farage and Long 1991) and *E. polyanthemos* (Holly *et al.* 1994).

Conclusion

Young *E. globulus* seedlings were found to be inherently susceptible to cold-induced photoinhibition. A severe photoinhibitory event can lead to photobleaching that significantly reduces seedling growth. The results indicated that young seedlings in a nursery should be protected by shadecloth in the winter at least until air temperatures favourable for growth are attained, particularly on days after low overnight temperatures followed by clear skies and bright sunlight.

Chapter 10. Summary and Implications for management

Introduction

Studies described in this thesis have investigated the physiological and growth responses of *Eucalyptus globulus* and *E. nitens* seedlings to cold-induced photoinhibition following various hardening treatments in the nursery and shading after planting. Two field trials at differing altitude and planting times and a controlled environment experiment were conducted. The overall aim was to measure the physiological changes that occurred in seedlings after planting and understand how seedlings acclimate to photoinhibition and photodamage in the field. This knowledge would form the basis for describing attributes necessary for the production of seedlings in the nursery that minimise mortality and maximise growth once planted.

Seedlings represent a major cost in plantation establishment and are more susceptible to abiotic and browsing stress relative to established saplings. Producing seedlings in the nursery that are optimally acclimated for survival as well as growth under conditions of cold-induced photoinhibition in the field is potentially complex. The current practice of raising seedlings under environmental conditions that, in relative terms, have high levels of nutrient and/or water supply, and transplanting them into distinctly different environments, involves risk. In contrast to a naturally regenerating seedling, a transplanted seedling does not have the physiological or physical attributes required for its new (planting) environment. This thesis has described in detail the physiological and physical responses of *E. globulus* and *E. nitens* during acclimation and establishment in the field. In doing so it provides baseline data to develop

optimal seedling specifications for seedling survival and performance during establishment.

Summary of results

Effects of hardening pre-treatments after early spring planting at 350 m asl (Chapter 3).

Seedling hardening pre-treatments were evaluated in an early spring planting on a site at 350 m asl that is considered marginal for planting *Eucalyptus globulus*, but optimal for planting *E. nitens*. Cold-induced photoinhibition and photodamage restricted growth and establishment of non-hardened *E. globulus*. These effects were mitigated by artificial shading. Artificial cold-hardening did not affect levels of cold-induced photoinhibition or growth of *E. nitens* seedlings. Artificial shading of *E. nitens* reduced cold-induced photoinhibition and photodamage but did not affect its growth. *E. nitens* seedlings hardened by nutrient starving were photoinhibited before planting and had high levels of anthocyanin. No increase in photoinhibition or photodamage occurred in nutrient-starved *E. nitens* after planting. In all other treatments the severity of cold-induced photoinhibition was related to level of increase of anthocyanin immediately after planting. *E. globulus* had relatively low levels of carotenoids compared to treatments other than NS *E. nitens*. In contrast to seedlings, established *E. nitens* saplings were not photoinhibited during the experimental period and had constant levels of anthocyanin.

Nitrogen and phosphorus fractions during establishment of* E. nitens *and* E. globulus *seedlings (Chapter 4)

Under favourable conditions in the nursery, superior growth rates of *E. globulus* were concluded to be due to greater acquisition of N and P. After planting, differences in soluble and labile inorganic P levels indicated that *E. globulus* has an inherent inability to acclimate photosynthetically to conditions of cold-induced photoinhibition. All treatments re-translocated N and P from shoots to roots soon after planting. Lower root:shoot ratios in *E. globulus* at planting required greater levels of re-translocation of N and P than *E. nitens*. Re-translocation increased root growth and root:shoot ratios relative to nursery values. Following this adjustment in root:shoot, N and P levels gradually increased and this was paralleled by increases in photosynthetic capacity and shoot growth. There was no re-translocation of nutrients in NS *E. nitens*. However rapid acquisition of N and P supported rapid recovery of photosynthetic capacity and growth rates similar to other treatments.

Effects of hardening pre-treatments after early winter planting at 700 m asl (Chapter 5)

Hardening treatments were evaluated at 700 m asl in early winter on a site considered marginal for establishment of *E. nitens*. Levels of NPQ higher than previously reported were linked to photooxidation of chlorophylls, xanthophylls and ß-carotene. This probably decreased light absorption. Concurrent increases in lutein and neoxanthin indicated their potential as antioxidants. There was a lack of correlation between PSII efficiency and xanthophyll cycle conversion state and this indicated that mechanisms other than the xanthophyll cycle were active for quenching excess light energy. Seedling growth was related to photosynthetic capacity, but not to

photochemical efficiency as measured by chlorophyll fluorescence. This discrepancy indicated light filtering in the upper cellular layers. Shading, by alleviating cold-induced photoinhibition, increased biomass production in winter but in spring and summer more biomass was produced in non-shaded seedlings. Shaded seedlings grew taller, probably due to enhanced apical dominance. Fertilised seedlings produced more biomass and grew taller in shaded than in non-fertilised treatments.

All treatments re-translocated nutrients from shoot to root after planting to increase root:shoot ratio. The greater levels of N and P re-translocated in fertilised treatments may have been a factor contributing to their greater biomass and height relative to non-fertilised seedlings.

Sustained xanthophyll activity is an overwintering strategy for* E. nitens *seedlings (Chapter 6)

Sustained xanthophyll cycle activity was identified in *E. nitens* treatments grown at 700 m asl. The recovery kinetics of PSII efficiency and xanthophyll cycle conversion state were characterised. Initial differences in their recovery furhter supports the contention that mechanisms other than the xanthophyll cycle were quenching excess light energy. Discrepancy between recovery kinetics of PSII efficiency and xanthophyll cycle conversion indicated independent recovery mechanisms. The severity of sustained xanthophyll activity was relatively less in fertilised and shaded compared to non-fertilised and non-shaded seedlings.

Physiological antioxidants and compounds deterring herbivory and in light attenuation? (Chapter 7)

Di-, tri-, tetra- and penta-galloylglucose and quercitin, rutin and other quercitin glycosides were identified in *E. nitens* foliage extracts sampled throughout the establishment period (in Chapter 3). These compounds varied in amounts relative to the levels of photoinhibition which varied with hardening treatment. The results were consistent with physiological antioxidant activity. Sideroxylonal levels increased in non-shaded treatments following herbivory by invertebrates in early summer. The kinetics of change in anthocyanin levels were distinct from those of galloylglucoses and flavonoids. Anthocyanin levels were greatest during the period of greatest severity of cold-induced photoinhibition. VIS-NIRS analysis indicated that anthocyanin increased absorption of light between 400 and 590 nm.

Effects of rapid induction of cold-induced photoinhibition (Chapter 8)

Severe cold-induced photoinhibition was rapidly induced under conditions of ~ 580 $\mu mol\ m^{-2}\ s^{-1}$ and 8 °C in a growth chamber. Rapid acclimation (within 2 days) involved photooxidation of xanthophylls and ß-carotene and synthesis of lutein and neoxanthin in non-fertilised treatments, and photooxidation of chlorophyll and xanthopyll synthesis in fertilised treatments. This was accompanied by a rapid increase in sustained xanthopyll cycle activity. The kinetics of xanthophyll cycle conversion state and PSII efficiency differed, consistent with results of Chapter 6. Significant synthesis of anthocyanins occurred after 8 days of cold-induced photoinhibition. The level of increase in anthocyanin was inversely related to the degree of total decrease in chlorophyll and directly related to the increase in xanthophyll pools. Levels of galloylglucoses, flavonoids and sideroxylonals

decreased rapidly following transfer to conditions of cold-induced photoinhibition. There was a lower requirement for antioxidants under conditions of increased sustained xanthopyll activity. It appeared that sideroxylonals are physiological antioxidants and not compounds which deter herbivory.

The re-translocation of nutrients following planting was from stored protein N and inorganic P. Growth and uptake of soil N decreased as indicated by decreased nucleic acid N and P and soluble N, respectively.

Cold-induced photoinhibition and photobleaching reduces growth of* Eucalyptus globulus *seedlings (Chapter 9)

Winter air temperatures >10 °C on clear mornings induced severe cold-induced photoinhibition and photodamage in *E. globulus* seedlings. This significantly reduced seedling growth compared to seedlings which were protected from photodamage by shading.

Implications of results

Seedling susceptibility to cold-induced photoinhibition and photodamage

Recently transplanted seedlings were inherently susceptible to cold-induced photoinhibition and photodamage. This was demonstrated by artificial shading which prevented severe cold-induced photoinhibition and photodamage of non-acclimated seedlings (Chapters 3, 5 and 9). Seedlings were shown to have low pigment levels and photosynthetic rates relative to foliage of acclimated saplings, characteristics which increase susceptibility to photoinhibition (Chapter 3). These effects are exacerbated as seedlings are in close proximity to the ground which increases

exposure of foliage to cold air stratification. Eucalypt plantations are often established on clear-felled coupes. Lack of overhead tree canopies increases the effects of radiative cooling on these sites (Nunez and Bowman 1986) and exposure to high levels of irradiance early in the morning. In environments of low temperature and high irradiance, cold-induced photoinhibition and photodamage are likely to be significant factors in the poorly defined phenomena of 'transplant shock' and 'transplant stress'.

***Cold-induced photoinhibition and tolerance of photodamage in* E. globulus *vs.* E. nitens**

The relative intolerance of *E. globulus* to frosts has been recognised for some time and is a major factor defining its distribution (Williams and Potts 1996). In contrast, the relative importance of cold-induced photoinhibition and photodamage has only recently been recognised as a factor affecting seedling regeneration and establishment. It has been shown that *E. globulus* is intolerant to cold-induced photoinhibition and photodamage relative to *E. nitens*. This is based on severe cold-induced photoinhibition, photodamage and growth depression of *E. globulus* in the nursery (Chapter 9) and after planting (Chapter 3) under conditions which have little of no effects on *E. nitens*. Physiological factors explaining these observations include the relatively low levels of carotenoids and synthesis of anthocyanins before planting (Chapter 3) and lack of ability to photosynthetically acclimate to cold conditions (Chapter 4) in *E. globulus* compared to *E. nitens*.

Effects on cold-induced photoinhibition and photodamage of various hardening pre-treatments

a) Artificial cold hardening

Cold hardening to low temperature is a process involving reduction of cell dehydration, minimisation of the build up of toxic solute concentrations and increased stability of plasma membranes (Steponkus 1984). It is important to note the distinction between these processes and changes in pigment (Chapters 5 and 8) and antioxidant (Chapters 7 and 8) levels in response to cold-induced photoinhibiton (Chapters 3, 5, 8 and 9). Artificial cold hardening can increase tolerance to potential damage by low temperature (Chapter 3) and increase tolerance to cold-induced photoinhibition and photodamage if coupled with exposure to moderate irradiance (Wanner and Junttila 1999). However, natural hardening and dehardening rapidly occurs below and above 9.5 °C, respectively, in tree seedlings (Greer *et al.* 2000) and the levels of hardening fluctuate according to seasonal temperatures (Chapter 3). Artificial cold-hardening had no effect on photodamage or growth of *E. nitens* seedlings under conditions of spring planting (Chapter 3). Thus, artificial night-time cold hardening of *E. nitens* is unlikely to be a practical hardening pre-treatment for dealing with cold-induced photoinhibition and photodamage in *E. nitens*.

b) Fertilising vs. nutrient starving

Seedling hardening to cold-induced photoinhibition and photodamage can be developed in the nursery via nutrient starving (Chapter 3). This practice is recommended for eucalypt seedlings being planted on cold sites. It relies on the ability of the seedlings to survive in a nutrient-deficient state and to grow rapidly once nutrients are available (Forest Research Institute 1987). Rapid recovery can be

accompanied by compensatory growth through rapid nutrient acquisition (Chapter 4). Photosynthetic and growth rates and biomass attained are then similar to those of non nutrient-starved seedlings (Chapter 3). This will occur on good quality sites planted in spring with adequate soil moisture and when temperatures are rapidly increasing. On high altitude sites it is necessary to plant before winter to avoid the risk of late spring frosts. Temperatures are decreasing and this restricts any compensatory growth by nutrient-starved seedlings (Chapter 5). The combination of higher photosynthetic rates (Chapters 5 and 8) and greater foliar nutrient availability for re-translocation for root growth (Chapters 4, 5 and 8) allow for more pre-winter growth in fertilised seedlings (Chapter 5).

Seedling risk management for cold-induced photoinhibition and photodamage vs. growth performance

The physiological effects of nutrient starving in the nursery and planting in early winter are, to some extent, similar. Photosynthetic rate is restricted, increasing photoinhibition (Chapters 3, 5 and 8). Within days (if changes in environmental conditions are rapid enough [Chapter 8]) photooxidation of light absorbing chlorophyll and ß-carotene pigments can occur in previously non-photoinhibited seedlings (Chapter 8), decreasing light absorption. Levels of xanthophylls, lutein, neoxanthin, galloylglucoses, sideroxylonals and flavonoids increase (Chapters 5, 7 and 8) and sustained xanthophyll cycle activity occurs (Chapter 6). If nutrient status restricts these changes (Chapters 5 and 8) or the severity of photoinhibition increases (Chapter 5), synthesis of anthocyanins occurs. Anthocyanins absorb, and thus attenuate, light otherwise absorbed by chlorophylls (Chapter 8). This re-balances light energy absorption with its utilisation and dissipation. The above processes

combine to prevent photodamage under conditions of severe cold-induced photoinhibition.

Nutrient starving, in inducing physiological processes which naturally occur during early winter, pre-hardens seedlings to photoinhibition and prevents photodamage which may occur out of season due to late or early frosts (eg. *E. globulus* in Chapter 3). However, unless conditions rapidly become favourable for growth (Chapter 3), growth of nutrient-starved seedlings is detrimentally affected by this treatment (Chapter 5). Given the rapid recovery of nutrient-starved seedlings once planted (Chapters 3 and 4), the effects of pre-hardening to photoinhibition are relatively short lived. The rapid acclimation and recovery of fertilised *E. nitens* seedlings planted in early spring on sites optimal for its establishment was demonstrated by similar growth between shaded and non-shaded *E. nitens*, where the latter sustained some photodamage (Chapter 3). Apparently late frosts of greater severity than those observed here, which induce greater levels of photodamage, are required for the potentially greater growth of nutrient-starved (which have proven resilience to photodamage [Chapter 3]) relative to fertilised *E. nitens* seedlings to be realised. Thus the decision to plant nutrient-starved or fertilised seedlings is a compromise between short term resilience to severe photodamage or greater initial growth coupled with natural hardening to cold-induced photoinhibition and photodamage. This decision will depend largely on the altitude and planting time of the site to be planted. Current and/or potential growth is best predicted using gas exchange rather than chlorophyll fluorescence (Chapter 5) due to leaf cell gradients of varying severity of photoinhibition.

Effects on cold-induced photoinhibition and photodamage of shadecloth shelters

Shadecloth tree shelters alleviated cold-induced photoinhibition of *E. globulus* and allowed growth rates similar to *E. nitens* (Chapter 3). This result, although demonstrating the extent to which cold-induced photoinhibition affects seedling establishment, is of little practical significance. *E. globulus* is unlikely to grow well at 350 m asl as, once seedlings grow above protective shadecloth shelters, its low foliar carotenoid levels (Chapter 3) and lack of ability to photosynthetically acclimate to low temperatures via alteration of P partitioning (Chapter 4), will limit its performance. However shadecloth protection is of practical benefit where very young seedlings developing in the nursery are highly susceptible to cold-induced photoinhibition and photodamage (Chapter 9) or at altitudes where *E. nitens* has the capacity to acclimate to ambient conditions of cold-induced photoinhibition but growth is limited by this process (Chapter 5). However, at 700 m asl superior growth during spring and summer in non-shaded seedlings offset the benefits of shading during winter. Spring and summer conditions would have to be less favourable for year round superior growth of shaded seedlings. Thus for forestry, shadecloth treeshelters are unlikely to be of practical use for increasing seedling biomass.

Shading increased height of seedlings over the growing season presumably via increased apical dominance. This may be desirable for increasing seedling height above stratified cold air layers or to escape the reach of vertebrate browsers. Given this result, cheaper shelters, which simply decrease perceived side-light relative to above-light, would produce the required effect on apical dominance.

Physiology of seedling nutrition during establishment

Increased metabolic activity (growth) and nutrient uptake can be maximised by maintaining higher ambient air and soil temperatures respectively (Chapter 8). These results, apart from the detail of the particular nutrient fractions involved, are readily understood by seedling producers. Management options for maximising soil and air temperatures include minimising excess water application. Also maintaining shadecloth covering during early morning and late afternoon in winter (during which time minimal photosynthesis occurs) as well as overnight will minimise radiative cooling.

Regardless of temperature, under identical conditions *E. globulus* will more efficiently acquire N and P relative to *E. nitens* (Chapter 4). This requires careful management of distinct nutrient application regimes for the two crops. *E. nitens*, with its relative lack of response to fertilisation requires higher levels of nutrients to attain reasonable growth rates. Conversely the efficient uptake of nutrient by *E. globulus* requires care not to over apply nutrient and maintain sensible root:shoot ratios.

A common response of both *E. globulus* and *E. nitens* to planting is the re-translocation of foliar N and P (Chapters 3 and 5) from storage forms (Chapter 8) for root growth which adjusts root:shoot ratios from nursery to field values (Chapters 4, 5 and 8). Greater levels of re-translocation occur in seedlings of lower ratio (Chapter 4). It is possible that this provides competition for N and/or P with processes of light energy utilisation and dissipation. If this was the case, seedlings of lower root:shoot, requiring greater re-translocation for adjustment are not desirable as they may experience greater severity of cold induced photoinhibition and photodamage soon

after planting. Options for managing this include maintaining root:shoot ratios close to field values in the nursery through careful nutrient application, which minimises seedling requirement for re-translocation soon after planting. Fertilising shortly before planting will maximise foliar nutrients available for re-translocation to roots.

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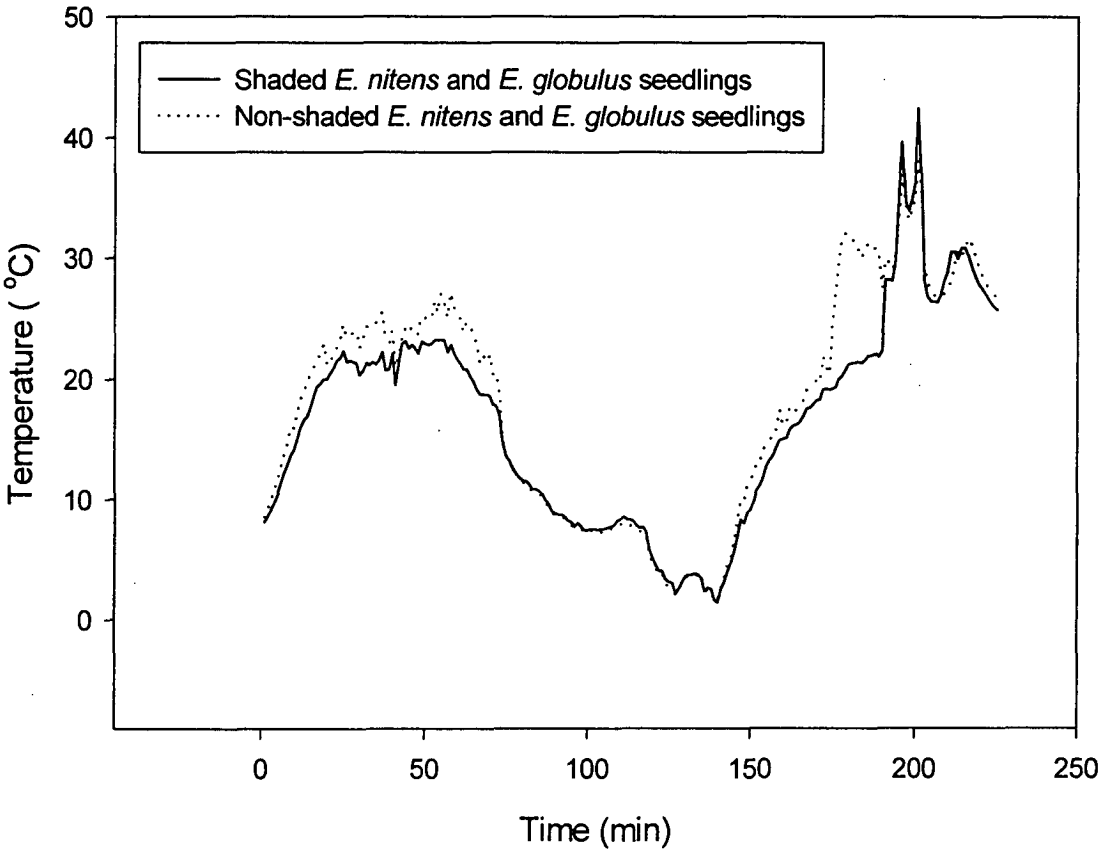
Appendix 1.

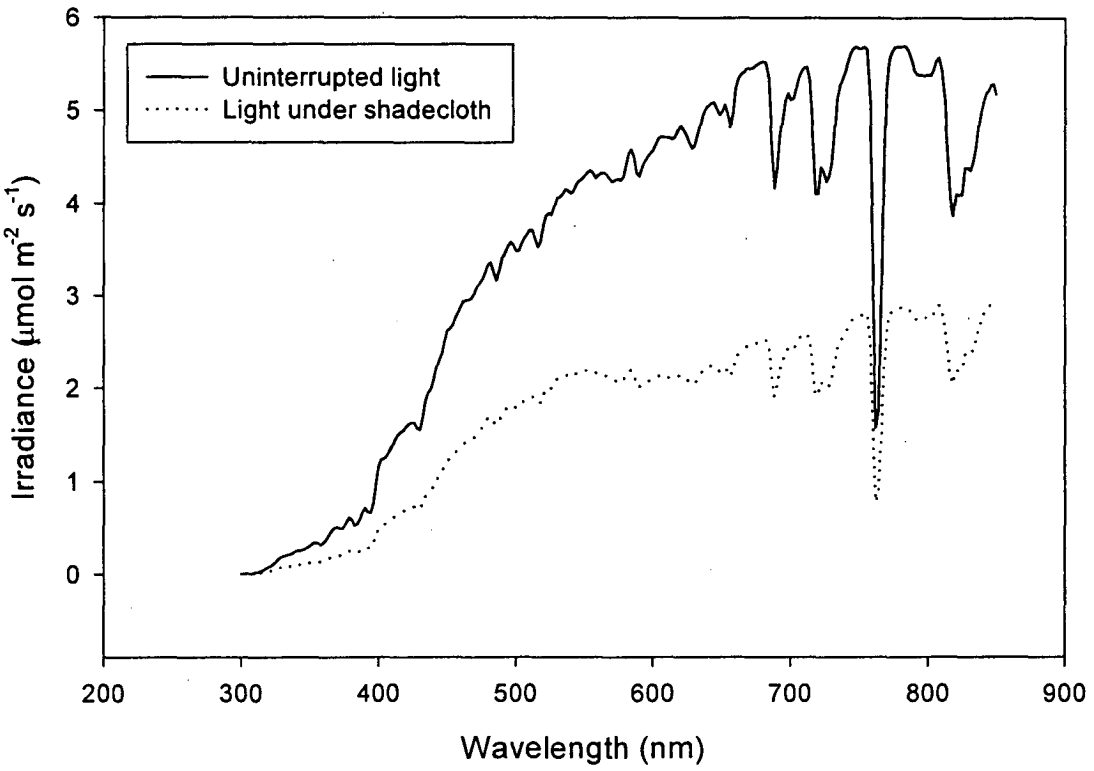
Air temperature at seedling height (30 cm) comparison of shaded vs. non-shaded seedlings during the Watsons block trial. Note the similarity of profiles when air temperature was > 8 °C. Each time unit on the x axis represents 10 mins.



Appendix 2.

Irradiance quantity and quality of uninterrupted light and light under shadecloth.

Irradiance was averaged for every 2 nm.



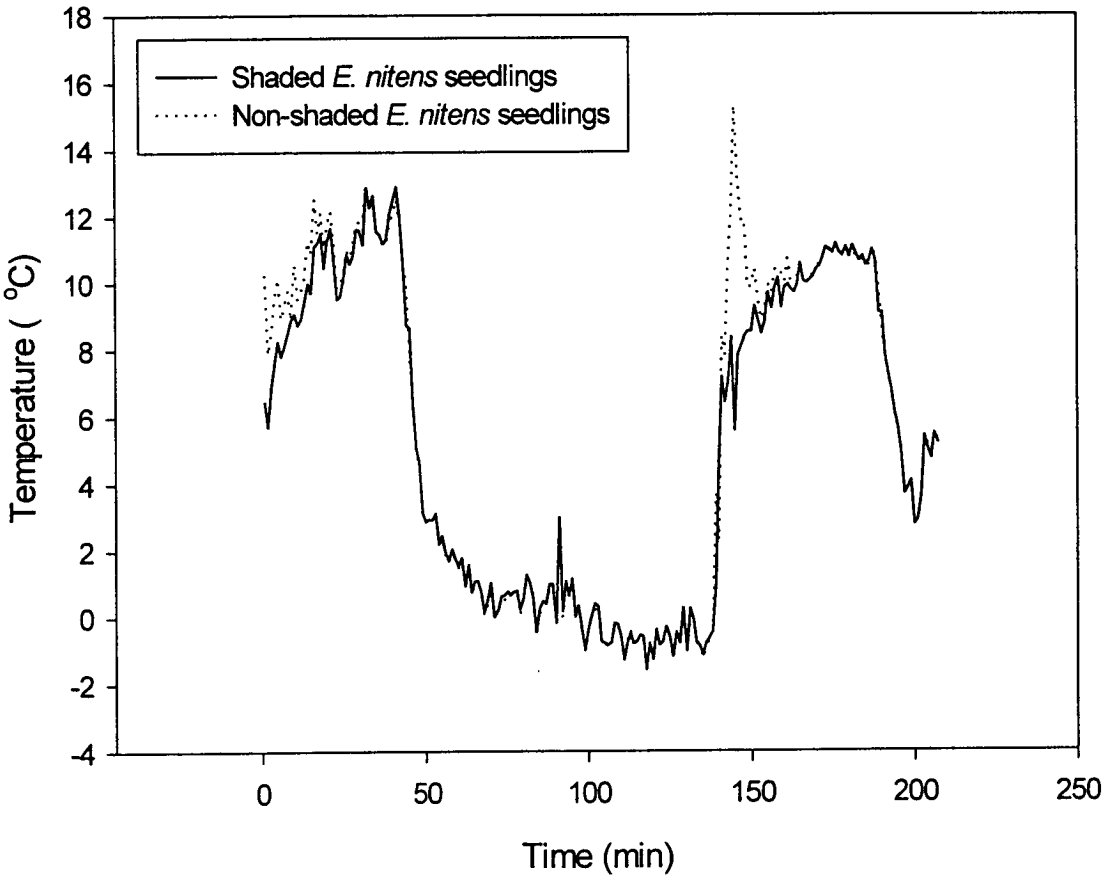
Appendix 3.

Air temperatures measured at 0.15, 0.30 and 0.45 m above ground level at two locations within the Watsons trial. Measurements are for the morning of September 19, 1997 following overnight frost. Sunrise was at 6.06 am.

Time	0.15 m	0.30 m	0.45 m	0.15 m	0.30 m	0.45 m
610	-0.381	-0.114	-0.049	-0.088	0.072	0.287
620	-0.218	0.066	0.191	0.019	0.178	0.419
630	0.692	0.838	0.998	0.666	0.662	0.761
640	1.349	1.52	1.649	1.568	1.503	1.486
650	2.287	2.458	2.514	2.548	2.463	2.484
700	3.568	3.833	3.735	4.06	3.598	3.696
Hourly Ave.	1.216167	1.4335	1.506333	1.462167	1.412667	1.522167
710	4.993	5.275	5.245	5.351	4.852	5.125
720	6.298	6.613	6.442	7.35	6.885	6.97
730	7.12	7.4	7.18	8.11	7.74	7.92
740	8.47	8.99	8.45	9.36	8.71	8.45
750	8.97	9.38	8.79	9.56	9.01	8.77
800	9.37	9.68	9.09	9.64	9.11	8.88
Hourly Ave.	7.536833	7.889667	7.532833	8.2285	7.717833	7.685833
810	9.77	10.14	9.47	10.37	9.83	9.58
820	10.52	10.81	10.23	10.49	9.83	9.47
830	10.8	11.01	10.33	10.73	10.12	9.72
840	11.06	11.32	10.93	11.25	10.75	10.54
850	10.61	10.75	10.21	11.11	10.57	10.28
900	11.34	11.6	11.1	12.2	11.46	11.12
Hourly Ave.	10.68333	10.93833	10.37833	11.025	10.42667	10.11833
910	11.18	11.45	10.96	11.21	10.61	10.37
920	11.15	11.36	10.77	11.69	10.9	10.52
930	12.07	12.22	11.48	12.36	11.62	11.32
940	12.11	12.4	11.73	12.19	11.46	11.25
950	11.54	11.55	10.94	12.07	11.34	10.9
1000	10.72	10.87	10.55	10.99	10.69	10.58
Hourly Ave.	11.46167	11.64167	11.07167	11.75167	11.10333	10.82333
1010	12.96	13.29	12.44	12.97	12.26	11.8
1020	12.25	12.47	11.83	12.92	12.14	11.72
1030	12.02	11.81	11.45	12.09	11.56	11.2
1040	12.02	11.9	11.55	11.82	11.55	11.11
1050	13.24	13.33	12.76	13.09	12.6	12.33
1100	13.06	12.71	12.37	13	12.55	11.92
Hourly Ave.	12.59167	12.585	12.06667	12.64833	12.11	11.68
1110	10.96	10.73	10.57	10.79	10.54	10.32
1120	10.92	10.76	10.55	10.9	10.68	10.43
1130	13.75	13.74	12.89	13.88	13.04	12.55
1140	12.02	11.65	11.32	12.23	11.75	11.37
1150	11.98	11.5	11.06	11.89	11.31	10.7
1200	11.73	11.41	11.19	11.57	11.3	11.04
Hourly Ave.	11.89333	11.63167	11.26333	11.87667	11.43667	11.06833

Appendix 4.

Air temperature at seedling height (30 cm) comparison of shaded vs. non-shaded seedlings during the Moory Rd. trial. Note the similarity of profiles when air temperature was > 8 °C. Each time unit on the x axis represents 10 mins.



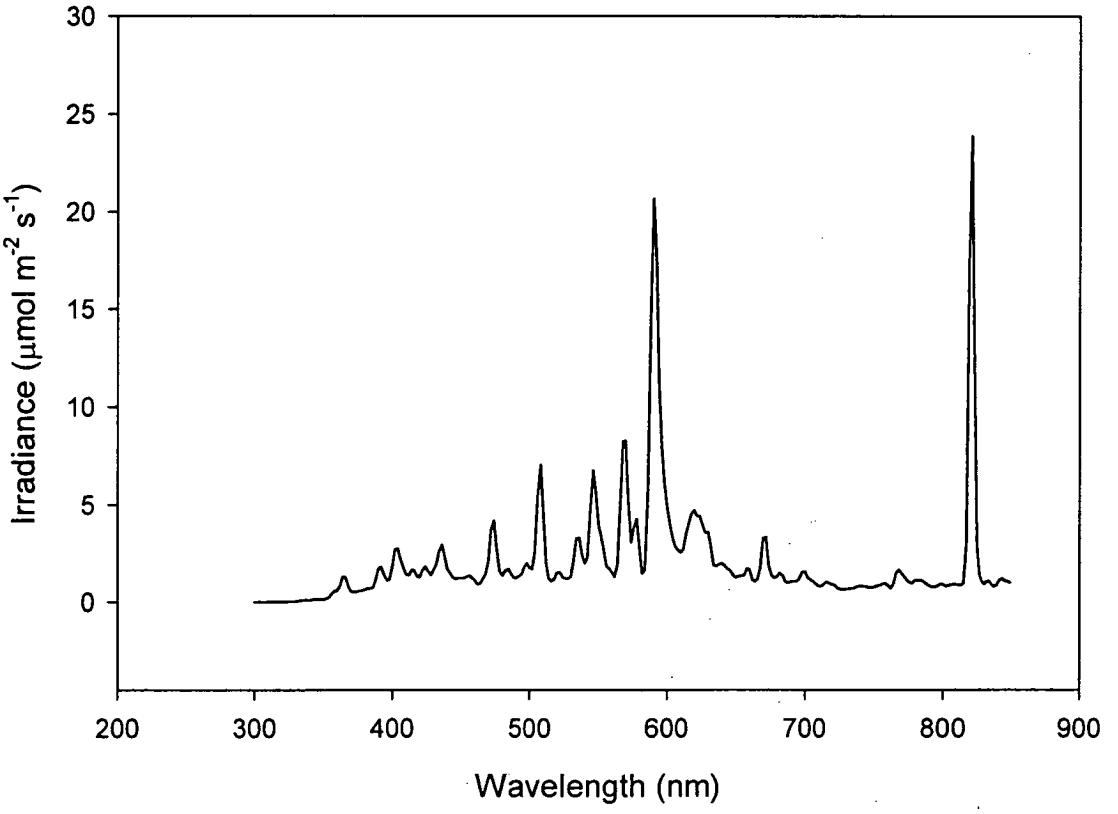
Appendix 5.

Sub-section of raw data gathered for one replicate and used for NPQ calculation (F_m/F_m'-1) reported for 1300 h of 13 August 1998 in Chapter 5. F_m values of 0.27475, 0.52208, 0.68475 and 0.76475 were averages of four replicates (of NF, F, Sh-NF and Sh-F respectively) and were measured pre-dawn. Unusually high NPQ values could be caused by the large amount of noise inherent in the very low fluorescence readings.

	No.	ML	Tmp.	PAR	Ft	Yield	Fm'	Fo'	NPQ
NF2									
	18	138	12.9	1211	0.003	0.667	0.008		
	19	138	12.1	555	0.013	0	0.009	0.008	33.34375
	20	138	11.9	840	0.014	0	0.01		
	21	138	11.9	376	0.006	0.444	0.011	0.009	26.475
F2									
	22	137	8.8	196	0.015	0.333	0.023		
	23	137	8.8	177	0.018	0.3	0.025	0.015	21.69913
	24	137	8	191	0.02	0.059	0.021		
	25	137	8.3	175	0.011	0.5	0.023	0.015	23.86095
SHNF2									
	26	139	7.6	129	0.023	0	0.021		
	27	139	7.8	143	0.016	0.278	0.023	0.015	31.60714
	28	139	7.8	222	0.024	0.136	0.028		
	29	140	8.4	418	0.031	0	0.028	0.018	23.45535
	30	139	11	1101	0.028	0	0.025		
	31	139	9.8	232	0.034	0	0.029	0.018	26.39
SHF2									
	32	138	8.5	154	0.028	0.389	0.045		
	33	137	8.6	150	0.03	0.415	0.051	0.024	15.99444
	34	137	8.3	129	0.034	0.4	0.056		
	35	137	8.8	148	0.03	0.467	0.056	0.023	12.65625

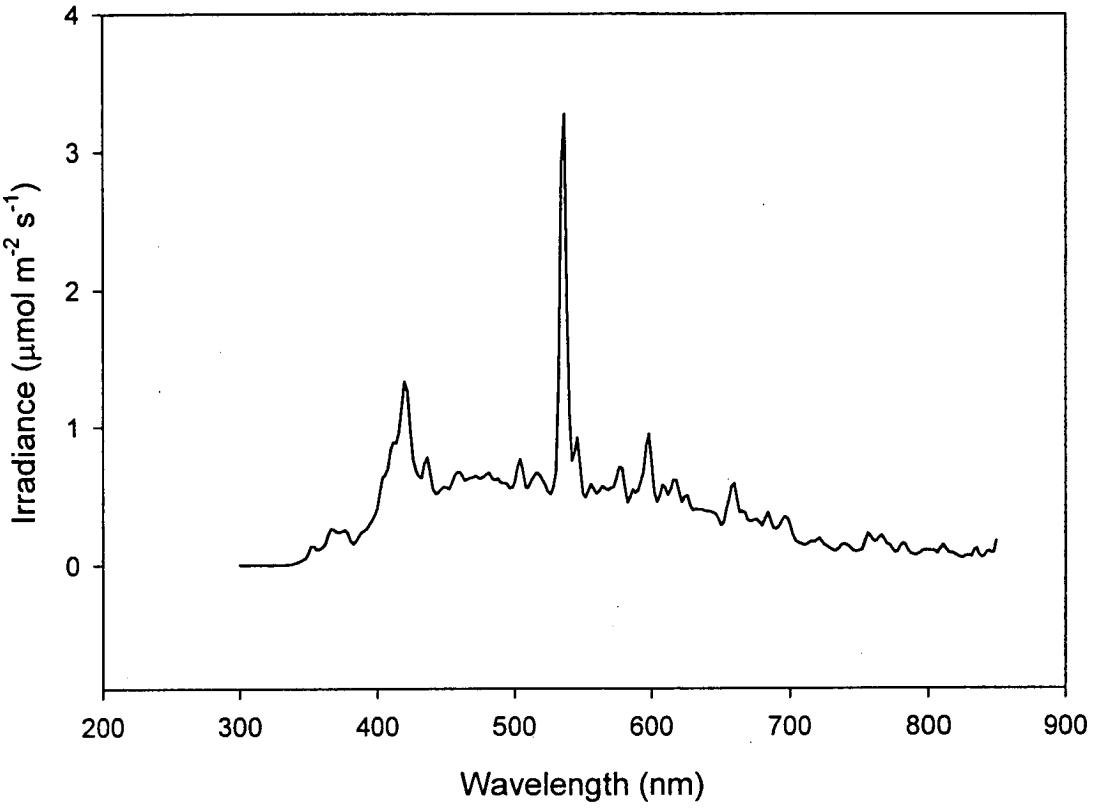
Appendix 6.

Irradiance quantity and quality of metal halide lamps used in Chapter 6. Irradiance was averaged for every 2 nm.



Appendix 7.

Irradiance quantity and quality of growth chamber light. Irradiance was averaged for every 2 nm.



Appendix 8.

Absolute values of ETR for NF, F, Sh-NF and Sh-F seedlings before and 17 days after GCT.

Before GCT

PAR	NF1	NF2	NF3	F1	F2	F3
10	2.8	3.2	3.7	3.7	3.2	3.7
50	10.9	14.6	12.2	18.1	16.1	17.5
120	19.6	23.9	16.4	42.3	34.5	41.7
210	28	31.7	17.1	66	54.1	60
350	37.7	40	20.4	95.7	76.1	78.7
500	23.6	29.6	18.5	113.1	73.8	100
700	22.6	100.6	52	153.5	84	121.8
950	42	59.7	40	172.1	130.9	146

17days after GCT

10	2.3	2.1	2.3	3.1	2.9	2.9
50	7.4	5.3	5.1	14.5	13.6	12.1
120	4.7	12.7	10.9	28.8	24.5	18.3
210	15.6	11.9	12.6	35	39.5	23
350	6.1	6.4	14.2	65.6	7.6	37.2
500	14	4.9	6.4	64.3	39.9	75
700	0	36.4	27.9	40.6	54.2	22.1
950	35.2	10.5	37.8	119.7	55.4	44.2

Before GCT

PAR	Sh-NF1	Sh-NF2	Sh-NF3	Sh-F1	Sh-F2	Sh-F3
10	3.2	3.4	3.5	3.4	4.1	3.7
50	10.5	17.7	18	16.2	17.5	13.9
120	14.5	32.5	36.7	34	37	24.7
210	14.5	43.4	62.8	49	61	41
350	7.1	56.3	84.5	68.4	69.5	48.9
500	37.2	62.6	103.8	52.9	69.4	67.9
700	36.8	63.2	112.3	57.2	40	129.5
950	83.9	71	167.1	83	93.3	49.2

17days after GCT

10	1.5	1.9	2.4	3.3	2.7	2.8
50	6.2	8.4	7.4	15.8	12.1	13.3
120	12.4	14.9	10.5	27.1	19	24.4
210	2.1	19.3	10.7	37.3	27.7	20
350	6.5	14.8	6.5	38.9	26.4	29.3
500	30.4	37.8	19.6	27.6	28.1	27.7
700	25.8	42.8	67.9	89.3	33.1	50.2
950	33.1	59.8	71.8	45.9	30.9	77.4